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(54) Title: USE OF A SELF–CLEAVING RNA MOTIF TO MODULATE GENE EXPRESSION

(57) Abstract

Methods for modulating expression of a desired nucleic acid product in a cell using a self–cleaving RNA motif are disclosed. Also disclosed are viral vectors and DNA constructs useful in methods for modulating production of a desired nucleic acid product *in vitro*, *in vivo* and *ex vivo*.

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USE OF A SELF-CLEAVING RNA MOTIF TO MODULATE GENE EXPRESSION

GOVERNMENT SUPPORT

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RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/111,579, filed December 9, 1998, and U.S. Provisional Application No. 60/105,472, filed October 23, 1998, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Methods for obtaining regulated expression of a product which are in common use today depend upon the expression of chimeric DNA binding/transcriptional transactivators and the use of specific hybrid promoter elements. One disadvantage of those strategies is that the expression of novel transactivator gene products in transduced cells, particularly in the case of *in vivo* applications, may result in a number of potential toxicities, including transcriptional activation or repression of endogenous genes, transcriptional squelching, or induction of immune responses directed towards the transactivator gene products. In addition, the need to incorporate both transactivator coding sequences and specialized promoter elements into transfer vectors places significant size constraints upon the foreign coding sequences which can subsequently be introduced into vectors which have a limited capacity for the insertion of foreign

sequences, such as adeno-associated virus (AAV). Further, proper gene regulation may be affected by the specific sites of chromosomal integration of the transcriptional cassettes, since endogenous transcriptional control elements adjacent to the inserted sequences may activate the expression of the desired gene in an uncontrollable way. Thus, there is a need to develop new and improved methods for regulated expression of a product which do not suffer from these limitations.

SUMMARY OF THE INVENTION

The present invention relates to the use of a self-cleaving RNA motif to modulate expression of a desired nucleic acid product in cells. Expression in cells in accordance with the present invention is modulated through the control of the cleavage of a messenger RNA (mRNA) which codes for the desired nucleic acid product; cleavage of the mRNA is controlled through the activity of a self-cleaving RNA motif which is located in the mRNA at a position such that the desired nucleic acid product is not expressed. Under conditions which permit expression of the self-cleaving RNA motif, the mRNA is cleaved and as a result, the desired nucleic acid product encoded is not produced. In the presence of an agent such as a drug (e.g., an antibiotic) or other molecule or composition, which inhibits (totally or partially) cleaving activity of the self-cleaving RNA motif, the desired nucleic acid product coded for by the mRNA is expressed. Modulation, as the term is used herein, includes induction, enhancement, reduction, inhibition (total or partial) and regulation. Regulation, as the term is used herein, refers to the ability to control the rate and extent to which a process occurs. For example, regulation of the activity of a self-cleaving RNA motif refers to the ability to control the rate and extent to which the activity of the self-cleaving RNA motif occurs. Regulation of expression of a desired nucleic acid product refers to the ability to control the rate and extent to which expression of the nucleic acid product occurs.

The cleaving activity of a self-cleaving RNA motif can be controlled by binding of an effector to an aptamer which is adjacent to the catalytic site of the self-cleaving RNA motif; an effector is a ligand which binds the aptamer, resulting in control of

cleaving activity of the self-cleaving RNA motif. Therefore, cleaving activity of the self-cleaving RNA motif can be modulated by binding of an effector to an aptamer which is adjacent to the catalytic site of the self-cleaving RNA motif. Thus, in cells present under conditions which permit (are appropriate for) expression of the desired nucleic acid product and the self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, binding of an effector to the aptamer results in modulation (induction, enhancement, reduction, inhibition (total or partial) or regulation) of the cleaving activity of the self-cleaving RNA motif. If the effector induces or enhances the cleaving activity of the self-cleaving RNA motif, the mRNA is cleaved and as a result, the desired nucleic acid product is not produced. If the effector reduces or inhibits the cleaving activity of the self-cleaving RNA motif, cleavage of the mRNA does not occur or is reduced, resulting in expression of the desired nucleic acid product. As used herein, an aptamer which is adjacent to the catalytic site of a self-cleaving RNA motif is located in a position such that the cleaving activity of the self-cleaving RNA motif can be modulated by binding of an effector to the aptamer.

The present invention provides DNA constructs comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed (a desired nucleic acid product) operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif. The DNA encoding the desired nucleic acid product and the DNA encoding the self-cleaving RNA motif are downstream of the promoter. The DNA encoding the self-cleaving RNA motif can be 5' of the DNA encoding the desired nucleic acid product or 3' of the DNA encoding the desired nucleic acid product. The term "promoter" refers to DNA which, when operably linked to DNA encoding a desired nucleic acid product, is sufficient for initiation of transcription of the DNA encoding the nucleic acid product to be expressed. Transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the self-cleaving RNA motif produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid

product. The cleaving activity of the self-cleaving RNA motif controls cleavage of the RNA molecule (mRNA) and, as a result, expression of the desired nucleic acid product; the self-cleaving RNA motif is located in the RNA molecule (mRNA) at a position such that the desired nucleic acid product is not expressed when the RNA molecule (mRNA) is cleaved. As used herein, a "nucleic acid product to be expressed" or "desired nucleic acid product" is a protein or polypeptide, DNA or RNA other than a self-cleaving RNA motif; it is also referred to herein as a nucleic acid product of interest. In a particular embodiment, the nucleic acid product to be expressed is a therapeutic protein.

The invention also provides DNA constructs comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed (a desired nucleic acid product) operably linked to the promoter; (c) DNA encoding a self-cleaving RNA motif, wherein the DNA of (b) and the DNA of (c) are downstream of the promoter; and (d) an intron which is 5' of the DNA encoding the nucleic acid product to be expressed. The intron can be upstream or downstream of the DNA encoding the self-cleaving RNA motif. Alternatively, the DNA encoding the self-cleaving RNA motif is present within the intron. In a particular embodiment, the DNA encoding the self-cleaving RNA motif is 5' of the DNA encoding the desired nucleic acid product. In another embodiment, the DNA encoding the self-cleaving RNA motif is 3' of the DNA encoding the desired nucleic acid product. Here, too, transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the self-cleaving RNA motif produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product, wherein the activity of the self-cleaving RNA motif controls cleavage of the RNA molecule and, as a result, expression of the desired nucleic acid product.

The present invention also provides DNA constructs comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed (a desired nucleic acid product), operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA

motif. The DNA encoding the desired nucleic acid product and the DNA encoding the self-cleaving RNA motif which includes the aptamer adjacent to its catalytic site are downstream of the promoter. The DNA encoding the self-cleaving RNA motif can be 5' of the DNA encoding the desired nucleic acid product or 3' of the DNA encoding the desired nucleic acid product. A self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif is also referred to herein as an "aptamer-self-cleaving RNA motif complex". Transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the aptamer-self-cleaving RNA motif complex produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex (which is mRNA) and mRNA encoding the desired nucleic acid product. As discussed above, the aptamer of the complex is in a position such that the cleaving activity of the self-cleaving RNA motif can be modulated by binding of an effector to the aptamer. The activity of the self-cleaving RNA motif controls cleavage of the RNA molecule and, as a result, expression of the desired nucleic acid product; the self-cleaving RNA motif is located in the RNA molecule at a position such that the desired nucleic acid product is not expressed when the RNA molecule is cleaved.

The invention further provides DNA constructs comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, wherein the DNA of (b) and the DNA of (c) are downstream of the promoter; and (d) an intron which is 5' of the DNA encoding the nucleic acid product to be expressed. The intron can be upstream or downstream of the DNA encoding the self-cleaving RNA motif. Alternatively, the DNA encoding the self-cleaving RNA motif can be present within the intron. The DNA encoding the self-cleaving RNA motif can be 5' or 3' of the DNA encoding the desired nucleic acid product. Transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the aptamer-self-cleaving RNA motif complex produces a RNA

molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product, wherein the activity of the self-cleaving RNA motif controls cleavage of the RNA molecule and, as a result, expression of the desired nucleic acid product.

In an alternative embodiment, the DNA constructs described above do not comprise a DNA encoding a self-cleaving RNA motif, but comprise DNA encoding a RNA motif that, when bound to another site on the mRNA transcript, results in cleavage of the transcript at the site bound by the RNA motif.

The invention relates to viral vectors which comprise the DNA constructs or the encoded (reverse transcribed) RNA, as well as to viral vectors, such as retroviral vectors, which represent the DNA construct. Other examples of viral vectors include adeno-associated viruses, adenoviruses, retroviruses, lentiviruses and herpesviruses.

The invention relates to packaging cell lines useful for generating recombinant viral vectors comprising a recombinant genome which includes a nucleotide sequence (RNA or DNA) which represents a DNA construct of the present invention. It also relates to construction of such cell lines and to methods of using the recombinant viral vectors to modulate production of a desired product *in vitro*, *in vivo* and *ex vivo*. In a particular embodiment, the recombinant viral vectors comprise a recombinant genome which includes a nucleotide sequence encoding a self-cleaving RNA motif, a nucleotide sequence encoding a desired nucleic acid product and a promoter operably linked to the nucleotide sequence encoding the desired nucleic acid product. In another embodiment, the recombinant viral vectors comprise a recombinant genome which includes a nucleotide sequence encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, a nucleotide sequence encoding a desired nucleic acid product and a promoter operably linked to the nucleotide sequence encoding the desired nucleic acid product. Transcription of the nucleotide sequence encoding the desired nucleic acid product and the nucleotide sequence encoding the self-cleaving RNA motif or the aptamer-self-cleaving RNA

motif complex produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif or aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product, wherein the activity of the self-cleaving RNA motif controls cleavage of the RNA molecule and, as a result, expression of the desired nucleic acid product.

Cell lines useful for generating recombinant viral vectors comprising a recombinant genome which includes a nucleotide sequence which represents a DNA construct of the present invention are produced by transfecting host cells, such as mammalian host cells, with a viral vector including the DNA construct integrated into the genome of the virus. The recombinant viral vectors produced by the packaging cell lines of the present invention are also referred to herein as viral vectors which represent the DNA construct.

The invention relates to a method of inducing expression of a desired nucleic acid product in a cell comprising introducing into the cell a DNA construct or a viral vector which represents the DNA construct. An agent which is capable of inhibiting cleavage of the self-cleaving RNA motif is subsequently introduced into the cell; inhibiting cleavage of the self cleaving RNA motif results in expression of the desired nucleic acid product.

The invention also relates to a method of modulating expression of a desired nucleic acid product in a cell comprising introducing into the cell a DNA construct or a viral vector which represents the DNA construct, wherein the DNA construct comprises (a) a promoter; (b) DNA encoding a desired nucleic acid product operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif. The DNA of (b) and the DNA of (c) are downstream of the promoter. Transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product. An effector which can bind the aptamer is introduced into the cell. Depending upon the design of

the aptamer-self-cleaving RNA motif complex, the cleaving activity of the self-cleaving RNA motif can be induced or enhanced and, as a result, the desired nucleic acid product is not produced, or the cleaving activity of the self-cleaving RNA motif can be reduced or inhibited and, as a result, the desired nucleic acid product is produced. In a particular embodiment, the DNA construct further comprises an intron which is 5' of the DNA encoding the nucleic acid product to be expressed.

The present invention also relates to a method of expressing or modulating expression of a desired nucleic acid product in an individual (e.g. a human or other mammal or vertebrate). The method comprises modulating expression of a nucleic acid product of interest from a DNA construct or a viral vector which represents the DNA construct, which is present in (contained in) cells in the individual. The DNA construct comprises DNA encoding the desired nucleic acid product and DNA encoding a self-cleaving RNA motif whose activity can be, in turn, modulated by an agent introduced into the cells when the desired nucleic acid product is to be expressed. Transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the self-cleaving RNA motif produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product.

In one embodiment, expression of a desired nucleic acid product is effected by administering an antibiotic to an individual, some of whose cells contain a DNA construct or viral vector representing a DNA construct of the present invention, wherein the DNA construct comprises (a) a promoter; (b) DNA encoding the desired nucleic acid product operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif, wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product. As a result, activity of the encoded self-cleaving RNA motif is inhibited (partially or totally), with the result that the nucleic acid product of interest is expressed in the individual. The DNA construct or viral vector which represents the DNA construct can be introduced into cells in the individual *in vivo* (e.g.,

by introducing the DNA construct or viral vector into a tissue or body fluid of the individual) or *ex vivo* (e.g., by introducing the DNA construct or viral vector into cells obtained from the individual or from another (different) individual or source and then introducing the resulting cells into the individual). In either case, administration of an antibiotic results in inhibition of the activity of the self-cleaving RNA motif and, as a result, the mRNA coding for the nucleic acid product of interest is not cleaved and the nucleic acid product of interest is expressed.

Generally, DNA will be introduced into cells through the use of viral vectors, such as DNA or RNA (retroviral) vectors. Retroviruses have been shown to have properties which make them particularly well suited to serve as recombinant vectors by which DNA of interest can be introduced into eukaryotic (e.g., mammalian, including human) cells. For example, recombinant retrovirus for use in gene transfer can be generated by introducing a suitable proviral DNA vector into fibroblastic cells that produce the viral proteins necessary for encapsidation of the desired recombinant RNA. This is one approach which can be used to introduce constructs of the present invention into mammalian, including human cells, for the purpose of modulation of gene expression. See, for example, Mann, R. *et al., Cell, 33*:153-159 (1983); Watanabe, S. and H.M. Temin, *Mol. Cell. Biol., 3*:2241-2249 (1983); Cone, R.D. and R.C. Mulligan, *Proc. Natl. Acad. Sci. USA, 81*:6349-6353 (1984); Soneoka, Y. *et al., Nucl. Acids Research, 123*:628-633 (1995); and Danos, O. and R.C. Mulligan, U.S. Patent No. 5,449,614. The DNA construct or viral vector in the individual's cells can, optionally, additionally comprise an intron, as described herein.

In a second embodiment, expression of a nucleic acid product of interest is effected by administering an aptamer-binding agent (effector) to an individual, some of whose cells contain a DNA construct or viral vector which represents a DNA construct of the present invention, wherein the DNA construct comprises (a) a promoter; (b) DNA encoding the desired nucleic acid product operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic

site of the self-cleaving RNA motif, wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product. The aptamer-self-cleaving RNA motif complex can be designed such that binding of the aptamer-binding agent to the aptamer results in reduction or inhibition (total or partial) of the catalytic activity of the self-cleaving RNA motif. As a result of binding of the aptamer-binding agent to the aptamer, activity of the self-cleaving RNA motif is reduced or inhibited, whereupon the desired nucleic acid product is expressed. Here, too, the DNA construct or viral vector can be introduced into cells in the individual *in vivo* or *ex vivo;* cells can be obtained from the individual (and returned to or reintroduced into the individual after the DNA construct or viral vector is introduced into them) or from another/different individual or source (and introduced into the individual after the DNA construct or viral vector is introduced). The DNA construct or viral vector in the individual's cells can, optionally, additionally comprise an intron, as described herein.

In one embodiment, the method is carried out by: (a) obtaining cells from an individual and maintaining the cells under appropriate conditions for cell growth and cell division; (b) introducing into the cells a DNA construct or viral vector representing a DNA construct of the invention; (c) returning the cells produced in step (b) to the individual; and (d) administering to the individual an agent which can inhibit cleavage of the self-cleaving RNA motif. In a particular embodiment, the DNA construct of the invention comprises (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif. The DNA encoding the nucleic acid product to be expressed and the DNA encoding the self-cleaving RNA motif are downstream of the promoter. In another embodiment, the DNA construct of the invention further comprises an intron which is 5' of the DNA encoding the nucleic acid product to be expressed. Transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the self-cleaving

RNA motif produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product. In this particular embodiment of the method of expressing a nucleic acid product in an individual, the agent is, for example, an antibiotic.

In another embodiment of the method of expressing a nucleic acid product in an individual, the method comprises: (a) obtaining cells from the individual and maintaining the cells under conditions appropriate for cell growth and cell division; (b) introducing into the cells a DNA construct or viral vector representing a DNA construct of the invention, wherein the DNA construct comprises (1) a promoter; (2) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; and (3) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, wherein the DNA of (2) and the DNA of (3) are downstream of the promoter, and transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product; (c) returning the cells produced in step (b) to the individual; and (d) administering to the individual an effector which can bind to the aptamer. In one embodiment, the DNA construct of the invention further comprises an intron which is 5' of the DNA encoding the nucleic acid product to be expressed. The effector (aptamer-binding agent) is any molecule which can bind the aptamer of the aptamer-self-cleaving RNA motif complex.

The invention further relates to a method of modulating expression of a desired nucleic acid product in an individual comprising: (a) introducing into the individual cells which comprise a DNA construct or viral vector which represents a DNA construct of the invention, wherein the DNA construct comprises (1) a promoter; (b) DNA encoding the desired nucleic acid product operably linked to the promoter; and (3) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, wherein the DNA of (2) and the DNA of (3) are downstream of the promoter, and transcription of the DNA of (2) and the DNA of (3)

produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product; and (b) administering to the individual an effector which can bind to the aptamer. As a result of binding of the effector to the aptamer, activity of the self-cleaving RNA motif is induced, enhanced, reduced, inhibited or regulated, depending upon the design of the self-cleaving RNA motif including the aptamer (aptamer-self-cleaving RNA motif complex) as discussed herein. If the cleaving activity of the self-cleaving RNA motif is induced or enhanced, the desired nucleic acid product is not produced. If the cleaving activity of the self-cleaving RNA motif is reduced or inhibited, the desired nucleic acid product is produced. The DNA construct or viral vector representing the DNA construct can be introduced into cells in the individual *in vivo* or *ex vivo* and cells can be obtained from the individual (and returned to or reintroduced into the individual after the DNA construct or viral vector is introduced into them) or from another/different individual or source (and introduced into the individual after the DNA construct or viral vector is introduced). The DNA construct or viral vector can, optionally, additionally comprise an intron, as described herein.

In one embodiment, the present invention relates to a method of regulating expression of an endogenous gene (a gene resident in a cell as the cell was obtained) to produce a desired nucleic acid product and compositions useful in the method. The endogenous gene can be one which is expressed ("on") in the cell or one which is normally not expressed ("off") in the cell but whose expression is or has been turned on or activated. In this embodiment, DNA encoding a self-cleaving RNA motif or a viral vector representing DNA encoding a self-cleaving RNA motif is introduced into genomic DNA of cells in such a position that, in mRNA produced by the cells, the self-cleaving RNA motif is in a location which results in control of expression of the encoded nucleic acid product. In the absence of an agent which can inhibit expression of the self-cleaving RNA motif, cleavage occurs and the desired nucleic acid product is expressed. In the presence of such an agent, cleaving activity is inhibited and the

desired nucleic acid product is expressed. In one embodiment, DNA encoding a self-cleaving RNA motif or a viral vector representing DNA encoding a self-cleaving RNA motif is introduced into genomic DNA between the promoter operably linked to (controlling expression of) the endogenous gene encoding the desired nucleic acid product, in such a manner that the endogenous gene remains operably linked to the promoter. In an alternative embodiment, DNA encoding a self-cleaving RNA motif or a viral vector representing DNA encoding a self-cleaving RNA motif is introduced into genomic DNA 3' of the endogenous gene encoding the desired nucleic acid product. The promoter which is operably linked to the endogenous gene to be expressed can be the naturally occurring (endogenous) promoter for the gene or can be an exogenous promoter introduced into genomic DNA. The resulting cells can be used, as described herein, to modulate production of the desired nucleic acid product in an individual.

The invention also relates to transgenic animals whose cells contain and express a DNA construct of the present invention. In a particular embodiment, a transgenic animal is produced by introducing into the germline of an animal or the germline of its ancestor, a DNA construct comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif, wherein the DNA of (b) and the DNA of (c) are downstream of the promoter, and transcription of the DNA of (b) and the DNA of (b) produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the nucleic acid product to be expressed. In another embodiment, the transgenic animal is produced by introducing into the germline of an animal or the germline of its ancestor, a DNA construct comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, wherein the DNA of (b) and the DNA of (c) are downstream of the promoter, and transcription of the DNA of (b) and the DNA of (b) produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and

mRNA encoding the nucleic acid product to be expressed. The DNA construct can further comprise an intron which is 5' of the DNA encoding the nucleic acid product to be expressed.

The invention further relates to transgenic plants whose cells contain a DNA construct of the present invention and express the encoded product. In a particular embodiment, the transgenic plant is produced by introducing into a plant a DNA construct comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif, wherein the DNA of (b) and the DNA of (c) are downstream of the promoter, and transcription of the DNA of (b) and the DNA of (b) produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the nucleic acid product to be expressed. In another embodiment, the transgenic plant is produced by introducing into a plant a DNA construct comprising (a) a promoter; (b) DNA encoding a nucleic acid product to be expressed, operably linked to the promoter; and (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the self-cleaving RNA motif, wherein the DNA of (b) and the DNA of (c) are downstream of the promoter, and transcription of the DNA of (b) and the DNA of (b) produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the nucleic acid product to be expressed. The DNA construct can further comprise an intron which is 5' of the DNA encoding the nucleic acid product to be expressed.

The invention relates to a method of identifying an effector which is capable of binding to a desired aptamer (or desired RNA sequence) comprising (a) introducing into host cells a DNA construct or a viral vector which represents the DNA construct, wherein the DNA construct comprises (1) a promoter; (2) DNA encoding a reporter operably linked to the promoter; and (3) DNA encoding a self-cleaving RNA motif which includes the desired aptamer (or desired RNA sequence) adjacent to the catalytic site of the self-cleaving RNA motif and such that binding of an effector to the aptamer

can inhibit the cleaving activity of the self-cleaving RNA motif, wherein the DNA of (2) and the DNA of (3) are downstream of the promoter, and transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex (or the desired RNA sequence-self-cleaving RNA motif complex) and mRNA encoding the reporter; (b) introducing into the host cells an agent to be assessed for its ability to bind the aptamer (or the desired RNA sequence) under conditions appropriate for expression of the reporter; and (c) assaying reporter activity in the host cells. If the agent binds to the aptamer (or the RNA sequence of interest), the cleaving activity of the self-cleaving RNA motif is inhibited and, as a result, the mRNA coding for the reporter is not cleaved and the reporter is produced. Therefore, if reporter activity is detected, the agent is identified as an effector which binds to the desired aptamer (or desired RNA sequence). A "desired aptamer" is also referred to herein as an aptamer of interest. A "desired RNA sequence" is also referred to herein as a RNA sequence of interest. A desired RNA sequence includes a desired aptamer.

The invention also relates to a method of screening for an agent which is capable of inhibiting the catalytic activity of a self-cleaving RNA motif including a random sequence at a position in the self-cleaving RNA motif capable of modulating the cleaving activity of the self-cleaving RNA comprising (a) introducing into host cells a DNA construct or viral vector which represents the DNA construct, wherein the DNA construct comprises (1) a promoter; (2) DNA encoding a reporter operably linked to the promoter; and (3) DNA encoding a self-cleaving RNA motif modified to include a random sequence at a position in the self-cleaving RNA motif capable of modulating the cleaving activity of the self-cleaving RNA, wherein the DNA of (2) and the DNA of (3) are downstream of the promoter; (b) introducing into the host cells an agent to be assessed for its ability to inhibit the catalytic activity of the self-cleaving RNA motif including the random sequence under conditions appropriate for expression of the reporter; and (c) assaying reporter activity in the host cells. If reporter activity is

detected, the mRNA coding for the reporter is not cleaved, indicating that the catalytic activity of the self-cleaving RNA motif including the random sequence is inhibited by the agent. In a particular embodiment, the random sequence is a random stem loop II.

The methods disclosed herein for modulating expression of a desired nucleic acid product do not require either the use of special transcriptional control elements or the expression of hybrid transactivator gene products. Thus, the methods of the present invention have a number of distinct advantages over previously developed methodologies for controlling expression of a desired nucleic acid product, and have broad application in the fields of protein production, gene therapy (e.g., human gene therapy), developmental biology, and functional genomics. In addition, the essential genetic element for gene regulation is very small in size and does not encode any gene product. Accordingly, it is unlikely that the introduction of the element into cells will result in any toxicity, and it should be possible to incorporate the necessary sequences for obtaining regulated expression into many different types of vectors.

An additional benefit of the methods described herein for modulating expression of a desired nucleic acid product is that gene regulation is not sensitive to chromosomal position, since modulation does not depend upon control of the initiation of transcription. Furthermore, in contrast to existing methods for controlling expression of a nucleic acid product, which require that specific hybrid promoters be used, it possible to modulate expression within the context of the normal cell type specific or developmental stage specific transcriptional elements of any gene or vector. In fact, by incorporation of the essential genetic element for gene regulation into introns within a transcriptional unit, it is even possible to provide gene regulation in the context of the normal mRNA structure used for gene expression (e.g., a structure devoid of any exogenous regulatory elements). These features may prove to be particularly important for transgenic and knockout experiments in animals designed to assess the role of a specific gene product at different stages of development, where the essential role of a

gene product in embryonal development may preclude the ability to determine the role of the gene product at a later stage of development.

In contrast to the case with those existing methods which make use of small molecules for gene regulation which have not been subject to the extensive pharmacological and/or toxicological testing necessary for approval for human use, particular methods and compositions described herein make use of standard antibiotics.

The invention also includes recombinant vectors which comprise the DNA constructs of the invention and host cells which comprise the DNA constructs and/or recombinant vectors of the invention. In addition to DNA encoding a nucleic acid product to be expressed, a promoter operably linked to the DNA encoding the nucleic acid product to be expressed and DNA encoding a self-cleaving RNA motif, vectors of the present invention can comprise additional DNA, such as an enhancer, targeting sequences, transcriptional binding sites and backbone DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A illustrates a naturally-occurring hammerhead ribozyme (HHRbz), a hammerhead ribozyme with a shorten stem loop II (HHRbz stII) and a mutated hammerhead ribozyme lacking cleaving activity (HHRbz Mut.)

Figures 1B-1C are schematic diagrams depicting the use of a self-cleaving RNA motif to modulate expression of a desired product.

Figure 2A is a schematic diagram of a pMD vector showing the insertion sites for a self-cleaving RNA motif. The indicated insertion sites are: between the HindIII-BamHI (A); at the MaeIII site (B); at the MboII site (C); at the SspI site (D); at the EcoRI site (E); and at the HaeIII site of the polyadenylation signal (poly A).

Figure 2B is a bar graph showing the effect of particular insertions in the pMD vector of a DNA encoding a self-cleaving RNA motif (HHRbz; Figure 1A) on expression of β-galactosidase in 293 cells transiently transfected with the pMD vector including the self-cleaving RNA motif. Restriction sites in the pMD vector at which a

self-cleaving RNA motif was inserted are indicated (A: between HindIII-BamHI; B: at MaeIII site; C: at MboII site; D: at SspI site; and E: at EcoRI site). "pMDA" indicates the control β-galactosidase activity, obtained by transient transfection with the control plasmid pMDA. "IVS" means intervening sequence.

Figures 3A-3B are two graphs showing induction of expression of β-galactosidase at various concentrations of chlortetracycline (Figure 3A) and at various concentrations of neomycin (Figure 3B) in 293T cells transiently transfected with a control plasmid (pMDA) or a pMD vector comprising DNA encoding a self-cleaving motif (HHRbz; Figure 1A) inserted between the HindIII-BamHI, at the MaeIII site, MboII site, SspI site or EcoRI site (see Figure 2A).

Figures 4A-4B are two bar graphs showing activation of expression of β-galactosidase at various concentrations of neomycin (Figure 4A) and at various concentrations of chlortetracycline (Figure 4B) in 293T cells transiently transfected with a control plasmid (pMDA nlslacZ) or a pMD vector comprising DNA encoding a self-cleaving RNA motif (HHRbz; Figure 1A) inserted between the HindIII-BamHI site or at the EcoRI site (see Figure 2A). The letters A and E indicate sites shown Figure 2A at which a self-cleaving RNA motic was inserted (A: between HindIII-BamHI; and E: at EcoRI).

Figures 5A-5B are two graphs comparing induction of expression of β-galactosidase at various concentrations of chlortetracycline in 293 cells transiently transfected with a control plasmid (pMDA), or a pMD vector comprising DNA encoding a self-cleaving RNA motif (HHRbz stII, HHRbz) or a mutated self-cleaving RNA motif lacking cleaving activity (HHRbz Mut.), inserted between the HindIII-BamHI site (see Figure 2A). A st. loop II: HHRbz stII; A: HHRbz; Mut: HHRbz Mut (see Figure 1A).

Figure 6 is a graph showing induction of expression of β-galactosidase at various concentrations of chlortetracycline in 293 cells transiently transfected with a control plasmid (pMDA), a pMD vector comprising DNA encoding a self-cleaving

RNA motif (HHRbz) or a mutated self-cleaving RNA motif lacking cleaving activity (HHRbz Mut.), inserted at the HaeIII site in the poly A site (see Figure 2A), or a pMD vector comprising DNA encoding a self-cleaving RNA motif (HHRbz) inserted beetween HindIII-BamHI (see Figure 2A).

Figure 7 is a graph showing the effect of stem loop II length on induction of expression of β-galactosidase at various concentrations of chlortetracycline in 293 cells transiently transfected with a control plasmid (pMDA), or a pMD vector comprising DNA encoding a self-cleaving RNA motif (HHRbz, HHRbz stII) or a mutated self-cleaving RNA motif lacking cleaving activity (HHRbz Mut.), inserted between the HindIII-BamHI site (see Figure 2A).

Figures 8A-8D are four bar graphs showing induction of expression of β-galactosidase at various concentrations of chlortetracycline (Figures 8A and 8C) and at various concentrations of demeclocycline (Figures 8B and 8D) in NIH 3T3 cells whose chromosome comprise a pMD vector comprising DNA encoding a self-cleaving RNA motif (HHRbz) inserted between the HindIII-BamHI site (see Figure 2A). These NIH 3T3 cell lines stably express pMD vector comprising DNA encoding the self-cleaving RNA motif.

Figures 9A-9B are schematic diagrams depicting the use of components of a self-cleaving RNA motif in producing a self-cleaving RNA motif for use in modulating expression of a product of interest.

Figure 10 is a schematic diagram of two approaches for identifying agents which modulate the activity of a self-cleaving RNA motif. The catalytic nucleotides are indicated by the shaded box.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of a self-cleaving RNA motif to modulate expression of a desired product in cells. Expression in cells in accordance with the present invention is modulated through the control of the activity of a self-

cleaving RNA motif which is located in the mRNA at a position such that the desired nucleic acid product is not expressed. Under conditions which are appropriate for expression of the self-cleaving RNA motif, the mRNA is cleaved and as a result, the desired nucleic acid product coded for by the mRNA is not produced (Figure 1B). Administration to cells of an agent such as a drug (e.g., an antibiotic) or other molecule or composition, which inhibits (totally or partially) cleaving activity of the self-cleaving RNA motif, prevents cleavage of the mRNA from occurring and the desired nucleic acid product is expressed (Figure 1C).

In one embodiment of the method of controlling expression of a desired nucleic acid product of the present invention, DNA encoding the desired nucleic acid product (which can be a polypeptide, DNA or RNA other than self-cleaving RNA) is expressed in cells as a component of a DNA construct which additionally comprises a promoter and DNA encoding a self-cleaving RNA motif. The DNA encoding the desired nucleic acid product is operably linked to the promoter. In another embodiment, a nucleotide sequence encoding the desired nucleic acid product is expressed in cells as a component of a recombinant viral vector comprising a recombinant genome which includes (a) the nucleotide sequence encoding the desired nucleic acid product, (b) a nucleotide sequence encoding a self-cleaving RNA motif and (c) a promoter operably linked to the nucleotide sequence encoding the desired nucleic acid product. Transcription of the two DNA or nucleotide components produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product.

If the DNA construct or viral vector is present in cells under conditions which permit expression of the two DNA or nucleotide components, the RNA molecule comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product is produced, the encoded self-cleaving RNA motif is spontaneously cleaved and, as a result, the nucleic acid product is not produced.

If, however, the DNA construct or viral vector is present in cells in the presence of an agent, such as a drug (e.g., an antibiotic) or other molecule or composition, which

inhibits (totally or partially) cleaving activity of the encoded self-cleaving RNA motif, the desired nucleic acid product is produced.

A self-cleaving RNA motif is capable of catalyzing cleavage (cleaving activity) in an intramolecular (cis) reaction, for example, at a specific site. The specific site at which cleavage can occur is in the same mRNA which also comprises the mRNA encoding the desired nucleic acid product. Self-cleaving RNA motifs include naturally-occurring ribozymes, such as the hammerhead, hairpin and hepatitis delta virus (HDV) ribozymes, ribozymes from plant pathogens, viroids, derivatives and modified forms of the naturally-occurring ribozymes, and synthetic ribozymes. These specific motifs are not limiting in the present invention and those skilled in the art will recognize that a self-cleaving RNA motif of the invention is any motif which catalyzes cleavage in an intramolecular (cis) reaction, as described herein.

DNA encoding a self-cleaving RNA motif of the present invention can be manufactured according to methods generally known in the art. For example, nucleic acid encoding a self-cleaving RNA motif can be manufactured by chemical synthesis or recombinant DNA/RNA technology (see, e.g., Sambrook *et al.*, Eds., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor University Press, New York (1989); and Ausubel *et al.*, Eds., *Current Protocols In Molecular Biology*, John Wiley & Sons, New York (1997)).

Cleavage of a self-cleaving RNA motif is sensitive to RNA sequences adjacent to the catalytic site of the self-cleaving RNA motif. Cleaving activity of the self-cleaving RNA motif can be modulated by binding of an effector to an aptamer which is adjacent to the catalytic site of the self-cleaving RNA motif. Thus, in cells present under conditions which are appropriate for expression of the desired nucleic acid product and the self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, binding of an effector to the aptamer results in modulation (induction, enhancement, reduction, inhibition (total or partial), or regulation) of the cleaving activity of the self-cleaving RNA motif. If the effector

induces the cleaving activity of the self-cleaving RNA motif, the mRNA is cleaved and as a result, the desired nucleic acid product is not produced. If the effector reduces or inhibits the cleaving activity of the self-cleaving RNA motif, cleavage of the mRNA does not occur or is reduced and the desired nucleic acid product is expressed. In the present invention, an aptamer which is adjacent to the catalytic site of a self-cleaving RNA motif is located in a position such that the cleaving activity of the self-cleaving RNA motif can be modulated by binding of an effector to the aptamer. A self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif is also referred to herein as "an aptamer-self-cleaving RNA motif complex". As discussed above, the aptamer of the complex is in a position such that the cleaving activity of the self-cleaving RNA motif can be modulated by binding of an effector to the aptamer. The aptamer moiety of the complex can be joined to the self-cleaving RNA motif of the complex covalently and directly (without intervening sequence or component) or indirectly (e.g., via a linker).

In a second embodiment of the present method of controlling expression of the desired product of the present invention, DNA encoding the desired nucleic acid product (which can be a polypeptide, DNA or RNA other than self-cleaving RNA) is expressed in cells as a component of a DNA construct which comprises the DNA encoding the desired nucleic acid product, a promoter and DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif. The DNA encoding the desired nucleic acid product is operably linked to the promoter. Transcription of the DNA encoding the desired nucleic acid product and the DNA encoding the aptamer-self-cleaving RNA motif complex produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product.

In another embodiment, a nucleotide sequence encoding the desired nucleic acid product is expressed in cells as a component of a recombinant viral vector comprising a recombinant genome which includes (a) the nucleotide sequence encoding the desired

nucleic acid product, (b) a nucleotide sequence encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif and (c) a promoter operably linked to the nucleotide sequence encoding the desired nucleic acid product. Transcription of the nucleotide sequence encoding the desired nucleic acid product and the nucleotide sequence encoding the aptamer-self-cleaving RNA motif complex produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product.

The aptamer-self-cleaving RNA motif complex is subject to allosteric regulation, in which the catalytic activity of the self-cleaving RNA motif is modulated upon binding of an effector to the aptamer moiety of the complex. Allosteric regulation of the aptamer-self-cleaving RNA motif complex involves binding of an effector to the aptamer of the complex. Typically, an aptamer-self-cleaving RNA motif complex can be designed such that binding of an effector to the aptamer of the complex results in induction, enhancement, reduction or inhibition (total or partial) of the catalytic activity of the self-cleaving RNA motif of the complex. For example, an aptamer-self-cleaving RNA motif complex can be designed such that binding of an effector to the aptamer moiety of the complex results in reduction or inhibition of the cleaving activity of the self-cleaving RNA motif moiety of the complex. Alternatively, an aptamer-self-cleaving RNA motif complex can be designed such that binding of an effector to the aptamer moiety of the complex results in induction or enhancement of the cleaving activity of the self-cleaving RNA motif moiety of the complex. Effectors can be, for example, organic ligands and include cofactors, saccharides, synthetic and recombinant peptides and proteins. Examples of effectors include glucose, adenodine 5'-triphosphate (ATP), flavinmononucleotide, and theophylline.

Thus, if a DNA construct comprising DNA encoding an aptamer-self-cleaving RNA motif complex, or a viral vector comprising a recombinant genome which includes a nucleotide sequence encoding an aptamer-self-cleaving RNA motif complex, is present in cells under conditions appropriate for expression of the DNA or nucleotide

sequence encoding the desired nucleic acid product and the DNA or nucleotide sequence encoding the aptamer-self-cleaving RNA motif complex, as described herein, an effector which binds the aptamer moiety of the complex modulates expression of the DNA or nucleotide sequence encoding the nucleic acid product by inducing, enhancing, reducing, inhibiting or regulating the cleavage of the self-cleaving RNA motif, depending on the design of the aptamer-self-cleaving RNA motif complex.

In a particular embodiment, DNA encoding an aptamer-self-cleaving RNA motif complex is designed by (a) selecting a self-cleaving RNA motif in which cleaving activity is to be modulated; (b) selecting an aptamer which is capable of binding to a selected effector; and (c) producing DNA comprising DNA encoding the selected aptamer and DNA encoding the selected self-cleaving RNA motif. DNA encoding an aptamer-self-cleaving RNA motif complex of the present invention can be manufactured according to methods generally known in the art. The DNA of the construct can be produced as separate "components" (e.g., as DNA encoding the nucleic acid product to be expressed and DNA encoding self-cleaving RNA motif, which are than joined using known methods or can be produced as a single continuous unit. For example, the DNA encoding an aptamer-self-cleaving RNA motif complex of the present invention can be manufactured by chemical synthesis or recombinant DNA/RNA technology (see, e.g., Sambrook *et al.*, Eds., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor University Press, New York (1989); and Ausubel *et al.*, Eds., *Current Protocols In Molecular Biology*, John Wiley & Sons, New York (1998).

Also the subject of the present invention is a DNA construct useful in the present method of controlling expression of a desired nucleic acid product in a cell. In one embodiment, the DNA construct comprises: (a) DNA encoding a nucleic acid product to be expressed in the cell; and (b) DNA encoding a self-cleaving RNA motif. Transcription of the two DNA components in the construct yields a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the nucleic acid

product to be expressed. The construct components can be separated by intervening DNA, such as a linker, provided that the intervening DNA does not interfere with the ability of the cleaving activity of the encoded self-cleaving RNA motif to disrupt (cleave) the mRNA coding for the desired nucleic acid product, thereby inhibiting/blocking expression the desired nucleic acid product. This embodiment of the DNA construct can be introduced into appropriate recipient/host cells in such a manner that the construct integrates into host cell genomic DNA at a location which results in its being operably linked to a host cell promoter (DNA sufficient to initiate transcription) and, as a result, expressed under the control of the host cell machinery. If the host cell is maintained under conditions appropriate for expression of DNA in the host cell (including expression of the DNA of the introduced--and now integrated--DNA construct), the encoded desired nucleic acid product is not expressed because the self-cleaving RNA motif is produced and its activity results in disruption of resulting transcript (mRNA), which cannot subsequently be translated. As a result, the encoded nucleic acid product is not expressed. If the host cell which contains the DNA construct of this embodiment is maintained under conditions appropriate for expression of DNA in the host cell and in the presence of an antibiotic (which prevents activity of the encoded self-cleaving RNA motif), disruption of the resulting transcript does not occur and the encoded desired nucleic acid product is expressed. In this embodiment, in which the DNA construct integrates into host cell genomic DNA, the construct can comprise additional DNA which increases the extent to which the DNA construct integrates into host cell genomic DNA and/or targets or directs introduction of the construct to a specific genomic location. The construct of this embodiment can also include additional components, such as an enhancer and transcriptional binding sites.

In an alternative embodiment, the DNA construct further comprises DNA sufficient for initiation of transcription (such as a promoter) operably linked to the DNA encoding the desired nucleic acid product. In a particular embodiment, the DNA encoding the self-cleaving RNA motif is 5' of the DNA encoding the desired nucleic acid product. Thus, the order of the components in the construct (from 5' to 3') is:

promoter - DNA encoding self-cleaving RNA motif - DNA encoding the desired nucleic acid product. In a second embodiment, the DNA encoding the self-cleaving RNA motif is 3' of the DNA encoding the desired nucleic acid product. Thus, the order of the components in the construct (from 5' to 3') is: promoter- DNA encoding the desired nucleic acid product - DNA encoding self-cleaving RNA motif.

In another embodiment, the DNA construct comprises (a) DNA encoding a desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the desired nucleic acid product; (c) DNA encoding a self-cleaving RNA motif, wherein the DNA of (a) and the DNA of (c) are downstream of the promoter; and (d) an intron which is 5' of the DNA encoding the desired nucleic acid product. The intron can be upstream or downstream of the DNA encoding the self-cleaving RNA motif, or the DNA encoding the self-cleaving RNA motif can be present within the intron. In a particular embodiment, the intron has a 5'-TACTAAC-3' box. In addition, the DNA encoding the self-cleaving RNA motif can be either 5' or 3' of the DNA encoding the desired nucleic acid product. Transcription of the two DNA components in the construct yields a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product.

In yet another embodiment, the DNA construct comprises (a) DNA encoding a desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the desired nucleic acid product; and (c) DNA encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, wherein the DNA of (a) and the DNA of (c) are downstream of the promoter. The DNA of (c) can be either 5' or 3' of the DNA encoding the desired nucleic acid product. Transcription of the two DNA components yields a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex (which is mRNA) and mRNA encoding the desired nucleic acid product.

In a further embodiment, the DNA construct comprises (a) DNA encoding a desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the

desired nucleic acid product; (c) DNA encoding a self-cleaving RNA motif which includes an aptamer which is adjacent to the catalytic site of the self-cleaving RNA motif, wherein the DNA of (a) and the DNA of (c) are downstream of the promoter; and (d) an intron which is 5' of the DNA encoding the desired nucleic acid product. The intron can be upstream or downstream of the DNA of (c), or the DNA of (c) can be present within the intron. In a particular embodiment the intron can have a 5'-TACTAAC-3' box. The DNA of (c) can be either 5' or 3' of the DNA encoding the desired nucleic acid product. Here, also, transcription of the two DNA components in the construct produces a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product.

The invention also relates to the DNA constructs which do not comprise DNA encoding a self-cleaving RNA motif, but comprise DNA encoding a RNA motif that, when bound to another site on the same RNA transcript, results in cleavage of the mRNA at that site. In one embodiment, the DNA construct comprises: (a) DNA encoding a desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the desired nucleic acid product; and (c) DNA encoding a RNA motif which, when bound to another site on the same RNA transcript, results in cleavage at the site in the mRNA bound by the RNA motif. If the DNA construct of this embodiment is present in host cells under conditions appropriate for binding of the RNA motif to another site on the same transcript (mRNA), the encoded desired nucleic acid product is not expressed because of cleavage of the transcript at the site in the mRNA bound by the RNA motif. If the host cell which contains the DNA construct of this embodiment is maintained under conditions appropriate for binding of the RNA motif to another site on the same transcript and in the presence of an antibiotic (which prevents activity of the bound RNA motif), disruption of the resulting transcript does not occur and the encoded desired nucleic acid product is expressed. A schematic diagram of this embodiment of the invention is presented in Figures 9A and 9B.

In a second embodiment, the DNA construct comprises: (a) DNA encoding a desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the desired nucleic acid product; and (c) DNA encoding a RNA motif which, when bound to another site on the same RNA transcript, results in cleavage at that site, and which includes an aptamer at a position where binding of an effector to the aptamer can modulate the cleaving activity at the site in the mRNA bound by the RNA motif. If the DNA construct of this embodiment is present in host cells under conditions appropriate for binding of the RNA motif to another site on the same transcript, an effector which binds the aptamer can modulate the cleaving activity at the site in the mRNA bound by the RNA motif.

The invention relates to packaging cell lines useful for generating recombinant viral vectors comprising a recombinant genome which includes a nucleotide sequence (RNA or DNA) which represents a DNA construct of the present invention, to construction of such cell lines and to methods of using the recombinant viral vectors to modulate production of a desired nucleic acid product *in vitro*, *in vivo* and *ex vivo*. In a particular embodiment, the recombinant viral vectors comprise a recombinant genome which includes a nucleotide sequence encoding a self-cleaving RNA motif, a nucleotide sequence encoding a desired nucleic acid product and a promoter operably linked to the nucleotide sequence encoding the desired nucleic acid product, as described herein. In another embodiment, the recombinant viral vectors comprise a recombinant genome which includes a nucleotide sequence encoding a self-cleaving RNA motif which includes an aptamer adjacent to the catalytic site of the self-cleaving RNA motif, a nucleotide sequence encoding a desired nucleic acid product and a promoter operably linked to the nucleotide sequence encoding the desired nucleic acid product, as described herein. Transcription of the two nucleotide components in the recombinant genomes produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif or the aptamer-self-cleaving RNA motif complex and mRNA encoding the desired nucleic acid product. In a further embodiment, the recombinant viral vectors of the

present invention comprise a recombinant genome which additionally include an intron, as described herein.

In an alternative embodiment, the recombinant viral vectors of the invention comprise a recombinant genome which does not include a nucleotide sequence encoding a self-cleaving RNA motif, but includes a nucleotide sequence encoding a RNA motif that, when bound to another site on the same RNA transcript, results in cleavage of the mRNA at that site, as described herein.

Cell lines useful for generating recombinant viral vectors comprising a recombinant genome which includes a nucleotide sequence which represents a DNA construct of the present invention are produced by transfecting host cells, such as mammalian host cells, with a viral vector including the DNA construct integrated into the genome of the virus, as described herein. Viral stocks are harvested according to methods generally known in the art. See, e.g., Ausubel *et al.*, Eds., *Current Protocols In Molecular Biology*, John Wiley & Sons, New York (1998); Sambrook *et al.*, Eds., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor University Press, New York (1989); Danos and Mulligan, U.S. Patent No. 5,449,614; and Mulligan and Wilson, U.S. Patent No. 5,460,959, the teachings of which are incorporated herein by reference. The recombinant viral vectors produced by the packaging cell lines of the present invention are also referred to herein as viral vectors which represent the DNA construct.

As used herein, the terms "a nucleic acid product to be expressed", "desired nucleic acid product" and "protein of interest" are used interchangeably. They can be protein or polypeptide, DNA or RNA other than a self-cleaving RNA motif. In a particular embodiment, the product to be expressed is a therapeutic protein. Examples of therapeutic proteins include antigens or immunogens, such as a polyvalent vaccine, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC,

ApoAI, the LDL receptor, negative selection markers or "suicide proteins", such as thymidine kinase (including the HSV, CMV, VZV TK), anti-angiogenic factors, Fc receptors, plasminogen activators, such as t-PA, u-PA and streptokinase, dopamine, MHC, tumor suppressor genes such as p53 and Rb, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels, such as a calcium channel or a potassium channel, and adrenergic receptors.

The invention also relates to a method of identifying an effector which is capable of binding to a desired aptamer (RNA sequence) comprising (a) introducing into host cells a DNA construct or a viral vector representing the DNA construct, wherein the DNA construct comprises (1) DNA encoding a reporter, (2) a promoter operably linked to the DNA encoding the reporter, and (3) DNA encoding a self-cleaving RNA motif which includes the desired aptamer (a desired RNA sequence) adjacent to the catalytic site of the self-cleaving RNA motif and such that binding of an effector to the aptamer (RNA sequence) can inhibit the cleaving activity of the self-cleaving RNA motif; (b) introducing into the host cells an agent to be assessed for its ability to bind the aptamer (RNA sequence) under conditions appropriate for expression of the reporter; and (c) assaying reporter activity in the host cells. Transcription of the two DNA components in the construct produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif which includes the desired aptamer (RNA sequence) (as described above) and mRNA encoding the reporter. If the agent binds to the aptamer (RNA sequence), the cleaving activity of the self-cleaving RNA motif is inhibited and, as a result, the mRNA coding for the reporter is not cleaved and the reporter is produced. Thus, if reporter activity is detected, the agent is identified as an effector which binds to the desired aptamer (desired RNA sequence). A schematic diagram of this embodiment of the invention is presented in Figure 10.

Alternatively, the self-cleaving RNA motif which includes the desired aptamer (desired RNA sequence) can be designed such that binding of an effector to the aptamer (RNA sequence) results in induction or enhancement of the cleaving activity of the self-

cleaving RNA motif. In this embodiment, if the agent binds to the aptamer (RNA sequence), cleavage of the self-cleaving RNA motif occurs which results in cleavage of the mRNA coding for the reporter and, as a result, the reporter is not produced. Therefore, in this embodiment, if reporter activity is not detected, the agent is identified as an effector which binds to the desired aptamer (desired RNA sequence).

The term "reporter" refers to a protein or polypeptide whose activity can be readily and easily assayed using standard techniques. Examples of reporters include enzymes, such as β-galactosidase, β-glucoronidase, β-glucosidase, bacterial chloramphenicol acetyl transferase (CAT), luminescent molecules, such as green flourescent protein and firefly luciferase, and auxotrophic markers such as His3p and Ura3p. See, e.g., Ausubel, F.M. *et al., Current Protocols in Molecular Biology*, Chapter 9, John Wiley & Sons, Inc. (1998).

The present invention also relates to a method of screening for an agent which is capable of inhibiting the catalytic activity of a self-cleaving RNA motif including a random sequence at a position in the self-cleaving RNA motif capable of modulating the cleaving activity of the self-cleaving RNA, comprising (a) introducing into host cells a DNA construct or viral vector representing the DNA construct, wherein the DNA construct comprises (1) DNA encoding a reporter, (2) a promoter operably linked to the DNA encoding the reporter, and (3) DNA encoding a self-cleaving RNA motif modified to include a random sequence at a position in the self-cleaving RNA motif capable of modulating the cleaving activity of the self-cleaving RNA, wherein the DNA of (1) and the DNA of (3) are downstream of the promoter, and transcription of the DNA of (1) and the DNA of (2) yields a RNA molecule (mRNA) comprising the self-cleaving RNA motif including the random sequence and mRNA encoding the reporter; (b) introducing into the host cells an agent to be assessed for its ability to inhibit the catalytic activity of the self-cleaving RNA motif including the random sequence under conditions appropriate for expression of the reporter; and (c) assaying reporter activity in the host cells. If reporter activity is detected, the mRNA coding for the reporter is not cleaved,

indicating that the catalytic activity of the self-cleaving RNA motif including the random sequence is inhibited by the agent. In a particular embodiment, the random sequence is a random step loop II. A schematic diagram of this particular embodiment is presented in Figure 10.

Agents, such as drugs, chemical compounds, ionic compounds, organic compounds, organic ligands, including cofactors, saccharides, recombinant and synthetic peptides, proteins, peptoids, and other molecules and compositions, can be individually screened or one or more agents can be tested simultaneously for the ability to bind to a desired aptamer or for the ability to modulate the cleaving activity of a self-cleaving RNA motif in accordance with the methods described herein. Where a mixture of agents is tested, the agents selected by the methods described can be separated (as appropriate) and identified by suitable methods (e.g., PCR, sequencing, chromatography). The presence of one or more agents in a test sample which bind a desired aptamer or modulate the cleaving activity of a self-cleaving RNA motif can also be determined according to these methods.

Large combinatorial libraries of agents (e.g., organic compounds, recombinant or synthetic peptides, peptoids, nucleic acids) produced by combinatorial chemical synthesis or other methods can be tested (see e.g., Zuckerman, R.N. *et al., J. Med. Chem., 37*:2678-2685 (1994) and references cited therein; see also, Ohlmeyer, M.H.J. *et al., Proc. Natl. Acad. Sci. USA 90*:10922-10926 (1993) and DeWitt, S.H. *et al., Proc. Natl. Acad. Sci. USA 90*:6909-6913 (1993), relating to tagged compounds; Rutter, W.J. *et al.* U.S. Patent No. 5,010,175; Huebner, V.D. *et al.*, U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). The teachings of these references are incorporated herein by reference. Where agents selected from a combinatorial library carry unique tags, identification of individual agents by chromatographic methods is possible.

Chemical libraries, microbial broths and phage display libraries can also be tested (screened) for the presence of one or more agents which bind to a desired aptamer

or modulate the cleaving activity of a self-cleaving RNA motif in accordance with the methods herein.

DNA constructs and DNA encoding self-cleaving RNA motifs of the invention can be introduced into a cell by a variety of methods (e.g., transformation, transfection, direct uptake, projectile bombardment, using liposomes). In a particular embodiment, a DNA construct or DNA encoding a self-cleaving RNA motif of the invention is inserted into a nucleic acid vector, e.g., a DNA plasmid, virus or other suitable replicon (e.g., viral vector). Viral vectors include retrovirus, adenovirus, parvovirus (e.g., adeno-associated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e.g., influenza virus), rhabdovirus (e.g., rabies and vesicular stomatitis virus), paramyxovirus (e.g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double stranded DNA viruses including adenovirus, herpesvirus (e.g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e.g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D-type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J.M., Retroviridae: The viruses and their replication, *In Fundamental Virology*, Third Edition, B.N. Fields, *et al.*, Eds., Lippincott-Raven Publishers, Philadelphia, 1996). Other examples include murine leukemia viruses, murine sarcoma viruses, mouse mammary tumor virus, bovine leukemia virus, feline leukemia virus, feline sarcoma virus, avian leukemia virus, human T-cell leukemia virus, baboon endogenous virus, Gibbon ape leukemia virus, Mason Pfizer monkey virus, simian immunodeficiency virus, simian sarcoma virus, Rous sarcoma virus and lentiviruses. Other examples of vectors are described, for example, in McVey et al., U.S. Patent No. 5,801,030, the teachings of which are incorporated herein by reference.

Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. Such methods are described in more detail, for example, in

Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor University Press, New York (1989); Ausubel, *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1998); and Danos and Mulligan, U.S. Patent No. 5,449,614, the teachings of which are incorporated herein by reference.

As a particular example of the above approach, a DNA construct of the invention can be integrated into the genome of a virus that enters the cell. By infection of the cell, the components of a system which permit expression of the DNA encoding the desired nucleic acid product and the spontaneous cleavage of the corresponding mRNA, are introduced into the cell. Under appropriate conditions, spontaneous cleavage of the corresponding mRNA occurs and expression of the encoded product is inhibited.

Virus stocks consisting of recombinant viral vectors comprising a recombinant genome which includes a nucleotide (DNA or RNA) sequence which represents a DNA construct of the present invention, are produced by maintaining the transfected cells under conditions suitable for virus production (e.g., in an appropriate growth media and for an appropriate period of time). Such conditions, which are not critical to the invention, are generally known in the art. See, e.g., *Sambrook et al., Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor University Press, New York (1989); *Ausubel et al., Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1998); U.S. Patent No. 5,449,614; and U.S. Patent No. 5,460,959, the teachings of which are incorporated herein by reference. The resulting recombinant viral vectors can be used, as described herein, to modulate production of a desired nucleic acid product in vitro, in vivo and ex vivo.

Thus, the invention also relates to recombinant viral vectors comprising a recombinant genome which includes a nucleotide (DNA or RNA) sequence which represents a DNA construct of the present invention. Viral vectors which comprise the DNA constructs or the encoded (reverse transcribed) RNA are also the subject of the present invention.

A vector comprising a DNA construct can also be introduced into a cell by targeting the vector to cell membrane phospholipids. For example, targeting of a vector of the present invention can be accomplished by linking the vector molecule to a VSV-G protein, a viral protein with affinity for all cell membrane phospholipids. Such a construct can be produced using methods well known to those practiced in the art.

For inhibition of expression of the DNA encoding the desired nucleic acid product, the cell can be maintained under appropriate conditions (e.g., normal conditions for cell growth and cell division) for spontaneous cleavage of the corresponding mRNA comprising the self-cleaving RNA motif. Generally, the cells are maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media, which are not critical to the invention, are generally known in the art and include sources of carbon, nitrogen and sulfur. Examples include Dulbeccos modified eagles media (DMEM), RPMI-1640, M199 and Grace's insect media. The pH which can be selected is generally one tolerated by or optimal for growth of the cell.

The cleaving activity of a self-cleaving RNA motif can be inhibited (partially or totally) using an agent such as a drug (e.g., an antibiotic) or other molecule or composition, which inhibits (partially or totally) the cleaving activity of the self-cleaving RNA motif. Inhibition of spontaneous cleavage of the corresponding mRNA results in the efficient induction of the expression of the nucleic acid product of interest. Antibiotics that can be used to inhibit the cleaving activity of a self-cleaving RNA motif include aminoglycoside antibiotics, such as, but not limited to, neomycin B, ribostamycin, paromomycin, neamine, gentamicin, lincomycin, kanamycin, tobramycin, 6'-amino-6'-deoxykanamycin and 5'-epi-sisomicin; tetracyclines and their derivatives and analogs, such as, but not limited to, tetracycline, chlortetracycline, demeclocycline, chelocardin and 4-epi-anhydrochlortetracycline; peptide antibiotics, such as, but not limited to, viomycin, di-β-lysyl capreomycin IIA and tuberactinomycin A; and pseudodisaccharide antibiotics, such as, but not limited to,

2'-de-N-1-β-lysyllysinomicin, 3-epi-6'-de-C-methylfortimicin B and 3-epi-2'-N-1-β-lysyl-6'-de-C-methylfortimicin B. Other antibiotics that can be used to inhibit the cleaving activity of a self-cleaving RNA motif are known and described in the art. See, for example, Stage *et al.*, *RNA, 1*:95-101 (1995); Clouet-d'Orval *et al.*, *Biochem., 34*11186-11190 (1995); Murray and Arnold, *Biochem. J., 317*:855-860 (1996); Hermann and Westhof, *J. Mol. Biol., 276*:903-912 (1998); and Rogers *et al., J. Mol. Biol., 259*:916-925 (1996), the teachings of which are entirely incorporated herein by reference.

Agents and effectors can be introduced into a cell according to methods generally known in the art which are appropriate for the particular agent or effector and cell type. For example, agents and effectors can be introduced into a cell by direct uptake, microinjection, calcium phosphate precipitation, electroporation, infection, and lipofection. Such methods are described in more detail, for example, in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor University Press, New York (1989); and Ausubel, *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1998). Other suitable methods are also described in the art.

As used herein, a cell refers to a prokaryotic cell, such as a bacterial cell, or eukaryotic cell, such as an animal, plant or yeast cell. A cell which is of animal or plant origin can be a stem cell or somatic cell. Suitable animal cells can be of, for example, mammalian or avian origin. Examples of mammalian cells include human (such as HeLa cells), bovine, ovine, porcine, murine (such as embryonic stem cells), rabbit and monkey (such as COS1 cells) cells. The cell may be an embryonic cell, bone marrow stem cell or other progenitor cell. Where the cell is a somatic cell, the cell can be, for example, an epithelial cell, fibroblast, smooth muscle cell, blood cell (including a hematopoietic cell, red blood cell, T-cell, B-cell, etc.), tumor cell, cardiac muscle cell, macrophage, dendritic cell, neuronal cell (e.g., a glial cell or astrocyte), or pathogen-infected cell (e.g., those infected by bacteria, viruses, virusoids, parasites, or prions).

The cells can be obtained commercially or from a depository or obtained directly from an individual, such as by biopsy. The cells used can be obtained from an individual to whom they will be returned or from another/different individual of the same or different species. For example, nonhuman cells, such as pig cells, can be modified to include a DNA construct and then introduced into a human. Alternatively, the cell need not be isolated from the individual where, for example, it is desirable to deliver the vector to the individual in gene therapy.

The present invention also relates to a method of regulating expression of an endogenous gene (a gene resident in a cell as the cell was obtained) to produce a desired nucleic acid product and compositions useful in the method. The endogenous gene can be one which is expressed ("on") in the cell or one which is normally not expressed ("off") in the cell but whose expression is or has been turned on or activated. DNA encoding a self-cleaving RNA motif, or a viral vector comprising a recombinant genome which includes a nucleotide (RNA or DNA) sequence which represents DNA encoding a self-cleaving RNA motif, can be introduced into genomic DNA of cells in such a position that in mRNA produced by the cells, the self-cleaving RNA motif is in a location which results in control of expression of the encoded product. In the absence of an agent which inhibits expression of the self-cleaving RNA motif, cleavage occurs and the desired nucleic acid product is expressed. In the presence of such an agent, cleaving activity is inhibited and the desired nucleic acid product is expressed. In one embodiment, DNA encoding a self-cleaving RNA motif, or a viral vector comprising a recombinant genome which includes a nucleotide (RNA or DNA) sequence which represents DNA encoding a self-cleaving RNA motif, is introduced into genomic DNA between the promoter operably linked to (controlling expression of) the endogenous gene encoding the desired nucleic acid product, in such a manner that the endogenous gene remains operably linked to the promoter. In an alternative embodiment, the DNA encoding a self-cleaving RNA motif, or the viral vector, is introduced into genomic DNA 3' of the endogenous gene encoding the desired nucleic acid product. The

promoter which is operably linked to the endogenous gene to be expressed can be the naturally occurring (endogenous) promoter for the gene or can be an exogenous promoter introduced into genomic DNA. The resulting cells can be used, as described herein, to modulate production of the desired nucleic acid product in an individual.

Also the subject of the present invention are cells (host cells) which comprise a DNA construct or viral vector of the invention. Particular cells which comprise a DNA construct of the invention are discussed above.

In a particular embodiment, a DNA construct of the invention can be used to produce transgenic animals whose cells contain and express the DNA construct. There is a variety of techniques for producing transgenic animals of the present invention. For example, foreign nucleic acid can be introduced into the germline of an animal by, for example, introducing the additional foreign genetic material into a gamete, such as an egg. Alternatively, transgenic animals can be produced by breeding animals which transfer the foreign DNA to their progeny. It is also possible to produce transgenic animals by introducing foreign DNA into somatic cells from which an animal is produced. As used herein, the term "transgenic animal" includes animals produced from cells modified to contain foreign DNA or by breeding; that is, it includes the progeny of animals (ancestors) which were produced from such modified cells. As used herein, the term "foreign nucleic acid" refers to genetic material obtained from a source other than the parental germplasm. Preferably, the transgenic animals are derived from mammalian embryos. The term "mammalian", as defined herein, refers to any vertebrate animal, including monotremes, marsupials and placental, that suckle their young and either give birth to living young (eutharian or placental mammals) or are egg-laying (metatharian or nonplacental mammals). Examples of mammalian species include primates (e.g., monkeys, chimpanzees), rodents (e.g., rats, mice, guinea pigs) and ruminents (e.g., cows, pigs, horses).

Methods for acquiring, culturing, maintaining and introducing foreign nucleic acid sequences into recipient eggs for transgenic animal production are well known in

the art. See, for example, *Manipulating the Mouse Embryo: A Laboratory Manual*, Hogan *et al.*, Cold Spring Harbor Laboratory, New York (1986). Preferably, the DNA construct will be delivered into the embryo at a very early stage in development so that only a small frequency of the embryos are mosaic (e.g., an embryo in which integration of the foreign nucleic acid occurs after the one cell stage of development).

A DNA construct of the present invention can also be used to produce transgenic plants whose cells contain the DNA construct and express the encoded nucleic acid product. As used herein, the term "transgenic plant" refers to plants in which foreign nucleic acid has been introduced into the nuclear, mitochondrial or plastid genome of a plant. As used herein, the term "plant" is defined as a unicellular or multicellular organism capable of photosynthesis. This includes the prokaryotic and eukaryotic algae (including cyanophyta and blue-green algae), eukaryotic photosynthetic protists, non-vascular and vascular multicellular photosynthetic organisms, including angiosperms (monocots and dicots), gymnosperms, spore-bearing and vegetatively-reproducing plants. Also included are unicellular and multicellular fungi.

A transgenic plant can be produced by introducing a DNA construct of the present invention into a plant cell using techniques well known in the art. Exemplary techniques are discussed in detail in Gelvin *et al.*, "Plant Molecular Biology Manual", 2nd Ed., Kluwen Academic Publishers, Boston (1995), the teachings of which are incorporated herein by reference.

For example, for grasses such as maize, nucleic acid molecules of the invention can be introduced into a cell using, for example, microprojectile bombardment (see, e.g., Sanford, J.C., *et al.*, U.S. Patent No. 5,100,792). In this approach, isolated DNA of the invention are coated onto small particles which are then introduced into the targeted tissue (cells) via high velocity ballistic penetration. The transformed cells are then cultivated under conditions appropriate for the regeneration of plants, resulting in production of transgenic plants. Transgenic plants carrying a DNA construct of the invention are examined for the desired phenotype using a variety of methods including,

but not limited to, an appropriate phenotypic marker, such as antibiotic resistance or herbicide resistance, or visual observation of the time of floral induction compared to naturally-occurring plants.

A DNA construct of the invention, as described herein, can also be introduced into a plant cell by *Agrobacterium*-mediated transformation (see, e.g., Smith, R.H., *et al.*, U.S. Patent No. 5,164,310) or electroporation (see, e.g., Calvin, N., U.S. Patent No. 5,098,843), or by using laser beams (see, e.g., Kasuya, T., *et al.*, U.S. Patent No. 5,013,660) or agents such as polyethylene glycol (see, e.g., Golds, T. *et al.*, *Biotechnology*, 11:95-97 (1993)), and the like. A DNA construct of the invention, as described herein, can also be inserted into a nucleic acid vector (e.g. an episomal vector or a Ti plasmid vector), or virus or other suitable replicon (e.g., a viral vector), which can be present in a single copy or multiple copies. Viral vectors which can be introduced into plant cells include cauliflower mosaic virus, figwort mosaic virus, and tobacco mosaic virus.

The vector can be introduced into a plant cell using techniques well known in the art. The method of introduction into the plant cell is not critical to this invention.

The viral vectors and DNA constructs of the present invention can be used in methods of inducing expression of a desired nucleic acid product in an individual (e.g., a human or other mammal or vertebrate). In these methods, a viral vector or DNA construct of the present invention can be introduced into cells obtained from the individual. The cells can be migratory, such as a hematopoietic cell, or non-migratory, such as a solid tumor cell or fibroblast. After treatment in this manner, the resulting cells can be administered to (introduced into) the individual according to methods known to those practiced in the art. To induce expression of the nucleic acid product, an agent, such as a drug (e.g., an antibiotic), which is capable of inhibiting cleavage of the encoded self-cleaving RNA motif, can be administered to the individual according to methods known to those practiced in the art. However, where the DNA construct comprises an aptamer-self-cleaving RNA motif complex or the viral vector represents

such a DNA construct, to modulate expression of the nucleic acid product, an effector which is capable of binding to the aptamer moiety of the complex can be administered to the individual. Such a treating procedure is sometimes referred to as *ex vivo* treatment. *Ex vivo* therapy has been described, for example, in Kasid *et al., Proc. Natl. Acad. Sci. USA, 87*:473 (1990); Rosenberg *et al., N. Engl. J. Med., 323*:570 (1990); Williams *et al., Nature, 310*:476 (1984); Dick *et al., Cell, 42*:71 (1985); Keller *et al., Nature, 318*:149 (1985); and Anderson *et al.*, United States Patent No. 5,399,346.

In a particular embodiment, the viral vectors and DNA constructs of the present invention can be used in a method of expressing a desired nucleic acid product in an individual. In this method, cells which comprise a viral vector or a DNA construct of the present invention are introduced into an individual. An agent, such as a drug, which is capable of inhibiting cleavage of the encoded self-cleaving RNA motif, is then administered to the individual, in whom the viral vector or DNA encoding the desired nucleic acid product is expressed, resulting in production of the product. In a particular embodiment of this method, the viral vector represents a DNA construct which comprises (a) DNA encoding the desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the desired nucleic acid product; and (c) DNA encoding self-cleaving RNA motif. The DNA encoding the desired nucleic acid product and the DNA encoding self-cleaving RNA motif are downstream of the promoter. The DNA encoding the self-cleaving RNA motif can be 5' or 3' of the DNA encoding the desired nucleic acid product. That is, in one embodiment, the order of the components in the construct (from 5' to 3') is: promoter - DNA encoding the desired nucleic acid product - DNA encoding self-cleaving RNA motif. In a second embodiment, the order of the components in the construct (from 5' to 3') is: promoter - DNA encoding self-cleaving RNA motif - DNA encoding the desired nucleic acid product. In a further embodiment, the viral vector represents a DNA construct which further comprises an intron which is 5' of the DNA encoding the desired nucleic acid product. The intron can be upstream or downstream of the DNA encoding the self-cleaving RNA motif, or the DNA encoding

the self-cleaving RNA motif can be present within the intron. Transcription of the two DNA components in the construct produces a RNA molecule (mRNA) comprising the self-cleaving RNA motif and mRNA encoding the desired nucleic acid product.

Alternatively, in a method for expressing a desired nucleic acid product in an individual, a DNA construct or viral vector of the present invention can be administered directly to the individual. The mode of administration is preferably at the location of the target cells. The administration can be nasally or by injection. Other modes of administration (parenteral, mucosal, systemic, implant, intraperitoneal, oral, intradermal, transdermal (e.g., in slow release polymers), intramuscular, intravenous including infusion and/or bolus injection, subcutaneous, topical, epidural, buccal, rectal, vaginal, etc.) are generally known in the art. The DNA construct or viral vector can, preferably, be administered in a pharmaceutically acceptable carrier, such as saline, sterile water, Ringer's solution, and isotonic sodium chloride solution. An agent, such as a drug, which is capable of inhibiting cleavage of the encoded self-cleaving RNA motif, is then administered to the individual, in whom the DNA encoding the desired nucleic acid product is expressed, resulting in production of the product.

In another embodiment, the viral vectors and DNA constructs of the present invention can be used in a method of modulating expression of a desired nucleic acid product in an individual. In this method, cells which comprise a viral vector or DNA construct of the present invention are introduced into an individual. An effector which is capable of binding to the aptamer moiety of the aptamer-self-cleaving RNA motif complex is then administered to the individual, whereupon expression of the DNA encoding the desired nucleic acid product can be induced, enhanced, reduced, inhibited or regulated, depending upon the design of the complex as discussed above. In a particular embodiment of this method, the viral vector represents a DNA construct which comprises (a) DNA encoding the desired nucleic acid product; (b) a promoter operably linked to the DNA encoding the desired nucleic acid product; and (c) DNA encoding an aptamer-self-cleaving RNA motif complex (a self-cleaving RNA motif

which comprises an aptamer adjacent to the catalytic site of the self-cleaving RNA motif). The DNA encoding the desired nucleic acid product and the DNA encoding the aptamer-self-cleaving RNA motif complex are downstream of the promoter. The DNA encoding the aptamer-self-cleaving RNA motif complex can be 5' or 3' of the DNA encoding the desired nucleic acid product. Transcription of the two DNA components in the construct yields a RNA molecule (mRNA) comprising the aptamer-self-cleaving RNA motif complex (which is mRNA) and mRNA encoding the desired nucleic acid product. In a second embodiment of this method, the viral vector represents a DNA construct which further comprises an intron which is 5' of the DNA encoding the nucleic acid product to be expressed. The intron can be upstream or downstream of the DNA encoding the aptamer-self-cleaving RNA motif complex, or the DNA encoding the aptamer-self-cleaving RNA motif complex can be present within the intron.

Alternatively, in a method for modulating expression of a desired nucleic acid product in an individual, a DNA construct or viral vector of the present invention can be administered directly to the individual. As above, the mode of administration can be, preferably, at the location of the target cells. Similarly, the administration can be nasally or by injection. Other modes of administration (parenteral, mucosal, systemic, implant, intraperitoneal, oral, intradermal, transdermal (e.g., in slow release polymers), intramuscular, intravenous including infusion and/or bolus injection, subcutaneous, topical, epidural, buccal, rectal, vaginal, etc.) are generally known in the art. The DNA construct or viral vector can, preferably, be administered in a pharmaceutically acceptable carrier, such as saline, sterile water, Ringer's solution, and isotonic sodium chloride solution. An effector which is capable of binding to the aptamer moiety of the aptamer-self-cleaving RNA motif complex can then be administered to the individual, whereupon expression of the DNA encoding the desired nucleic acid product can be induced, enhanced, reduced, inhibited or regulated, depending upon the design of the complex as discussed above.

Agents and effectors can be administered to an individual in a variety of ways. The route of administration depends upon the particular agent or effector. Routes of administration are generally known in the art and include oral, intradermal, transdermal (e.g., in slow release polymers), intramuscular, intraperitoneal, intravenous including infusion and/or bolus injection, subcutaneous, topical, epidural, buccal, rectal, vaginal and intranasal routes. Other suitable routes of administration can also be used, for example, to achieve absorption through epithelial or mucocutaneous linings.

The dosage of agent, effector, DNA construct or viral vector of the present invention administered to an individual, including frequency of administration, will vary depending upon a variety of factors, including mode and route of administration; size, age, sex, health, body weight and diet of the recipient; nature and extent of symptoms of the disease or disorder being treated; kind of concurrent treatment, frequency of treatment, and the effect desired.

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLES

Figure 1A illustrates a naturally-occurring hammerhead ribozyme (HHRbz), a hammerhead ribozyme with a shorten stem loop II (HHRbz stII) and a mutated hammerhead ribozyme lacking cleaving activity (HHRbz Mut.). Stem loops I, II and III in each ribozyme are indicated. The site at which cleavage of the ribozyme occurs is indicated by the arrow. Differences between the mutated hammerhead ribozyme lacking cleaving activity (HHRbz Mut.) and the naturally-occurring ribozyme (HHRbz) is shown in bold. Differences between the naturally-occurring hammerhead ribozyme (HHRbz) and the hammerhead ribozyme with the shorten stem loop II (HHRbz stII) can be determined by comparing the nucleotide bases in the stem loop IIs of the ribozymes.

The pMD vector illustrated in Figure 2A contains the β-galactosidase gene (*nlslacZ*) operably linked to the CMV promoter (pCMV). 5' of the β-galactosidase gene is the intron (IVS) flanked by exon 2 (ex2) and exon 3 (ex3) of the human β-globulin gene and 3' of the β-galactosidase gene is a polyadenylation signal (poly A) from the human β-globulin gene (β-glob). This vector can be constructed as described in Ory, D.S. *et al., Proc. Natl. Acad. Sci. USA, 93*:11400-11406 (1996), the teachings of which are incorporated herein by reference. Restriction sites in the pMD vector at which a self-cleaving RNA motif was inserted are indicated (A: HindIII-BamHI site; B: MaeIII site; C: MboII site; D: SspI site; and E: EcoRI site). The HaeIII site of the polyadenylation signal at which a self-cleaving RNA motif was inserted is also indicated.

Figure 2B depict the results of experiments assessing the effect of insertion of a DNA encoding the naturally-occurring hammerhead ribozyme (HHRbz) in the pMD vector in human embryonic kidney 293 cells transiently transfected with a pMD vector comprising DNA encoding the ribozyme inserted between the HindIII-BamHI site, at the MaeIII site, MboII site, SspI site or EcoRI site. Insertion of the ribozyme at the HindIII-BamHI site and at the SspI site resulted in the greater relative degree of inhibition of β-galactosidase activity relative to the level of β-galactosidase activity produced by 293 cells transiently transfected with the control plasmid pMDA (Figure 2B).

Figures 3A and 3B depict the results of experiments assessing the induction of expression of β-galactosidase at various concentrations of chlortetracycline (Figure 3A) and at various concentrations of neomycin (Figure 3B) in 293T cells transiently transfected with a pMD vector comprising DNA encoding a naturally-occurring hammerhead ribozyme (HHRbz) inserted between the HindIII-BamHI site, at the MaeIII site, MboII site, SspI site or EcoRI site. Induction of expression of β-galactosidase at various concentrations of chlortetracycline was greatest in 293 cells transfected with the pMD vector comprising DNA encoding the ribozyme inserted

between the HindIII-BamHI site (Figure 3A). Induction of expression of β-galactosidase at various concentrations of neomycin was greatest in 293 cells transfected with the pMD vector comprising DNA encoding the ribozyme inserted between the HindIII-BamHI site or at the MaeIII site (Figure 3B).

Figures 4A and 4B depict the results of experiments assessing the activation of expression of β-galactosidase at various concentrations of neomycin (Figure 4A) and at various concentrations of chlortetracycline (Figure 4B) in 293T cells transiently transfected with a pMD vector comprising DNA encoding the naturally-occurring hammerhead ribozyme (HHRbz) inserted between the HindIII-BamHI site or at the EcoRI site. Activation of expression of β-galactosidase at various concentrations of neomycin and at various concentrations of chlortetracycline was greater in 293 cells transfected with the pMD vector comprising DNA encoding the ribozyme inserted between the HindIII-BamHI site than in 293 cells transfected with the pMD vector comprising DNA encoding the ribozyme inserted at the EcoRI site

Figures 5A and 5B depict the results of experiments assessing the effect the use of a hammerhead ribozyme with a shorten stem loop II has in modulating expression of β-galactosidase at various concentrations of chlortetracycline 293 cells transiently transfected with a pMD vector comprising DNA encoding a naturally-occurring hammerhead ribozyme (HHRbz stII) inserted between the HindIII-BamHI site, or with a pMD vector comprising DNA encoding a hammerhead ribozyme with a shorten stem loop II (HHRbz stII) inserted between the HindIII-BamHI site. β-galactosidase activity was induced at lower concentrations of chlortetracycline in 293 cells transfected with the pMD vector comprising DNA encoding the hammerhead ribozyme with the shorten stem loop II relative to 293 cells transfected with the pMD vector comprising DNA encoding the naturally-occurring hammerhead ribozyme, indicating that the ribozyme with the shorten stem loop II is more sensitive to regulation at lower concentrations of antibiotic. That is, less antibiotic is required for inhibition of the cleaving activity of the ribozyme.

Figure 6 depicts the results of an experiment assessing the induction of expression of β-galactosidase at various concentrations of chlortetracycline in 293 cells transiently transfected with a pMD vector comprising DNA encoding a naturally-occurring hammerhead ribozyme (HHRbz) inserted at the HaeII site in the poly A site. β-galactosidase activity induced in 293 cells transfected with the ribozyme was similar to the β-galactosidase activity in 293 cell transiently transfected with the control plasmid pMDA.

Figure 7 depicts the results of an experiment assessing the effect of stem loop II length on the induction of expression of β-galactosidase at various concentrations of chlortetracycline in 293 cells transiently transfected with a pMD vector comprising DNA encoding a hammerhead ribozyme with a shorten stem loop II (HHRbz stII), inserted at the HindIII-BamHI site. β-galactosidase activity was higher in 293 cells transfected with the pMD vector comprising DNA encoding the hammerhead ribozyme with the shorten stem loop II relative to 293 cells transfected with a pMD vector comprising DNA encoding a naturally-occurring hammerhead ribozyme (HHRbz), indicating that the activity of the ribozyme with the shorten stem loop II is more sensitive to regulation at lower concentrations of antibiotic. That is, less antibiotic is required for inhibition of the cleaving activity of the ribozyme.

Figures 8A-8D depict the results of experiments assessing the induction of expression of β-galactosidase at various concentrations of chlortetracycline (Figures 8A and 8C) and at various concentrations of demeclocycline (Figures 8B and 8D) in NIH 3T3 cells whose chromosome comprise a pMD vector comprising DNA encoding a hammerhead ribozyme (HHRbz) inserted between the HindIII-BamHI site. β-galactosidase activity was not detected in the cells in the absence of antibiotic. β-galactosidase activity was detected in the cells in the presence of antibiotic.

The teachings of all the articles, patents and patent applications cited herein are incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A DNA construct comprising:
   (a) a promoter;
   (b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and
   (c) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,
   wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly.

2. The DNA construct of Claim 1 further comprising an intron, wherein the intron is 5' of the DNA of (b).

3. The DNA construct of Claim 2 wherein the DNA of (c) is upstream of the intron.

4. The DNA construct of Claim 2 wherein the DNA of (c) is downstream of the intron.

5. The DNA construct of Claim 2 wherein the DNA of (c) is present in the intron.

6. A DNA construct comprising:
   (a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly.

7. The DNA construct of Claim 6 further comprising an intron, wherein the intron is 5' of the DNA of (b).

8. The DNA construct of Claim 7 wherein the DNA of (c) is upstream of the intron.

9. The DNA construct of Claim 7 wherein the DNA of (c) is downstream of the intron.

10. The DNA construct of Claim 7 wherein the DNA of (c) is present in the intron.

11. A recombinant vector comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly.

12. A recombinant viral vector comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter;

(c) DNA encoding a self-cleaving RNA motif which is downstream of said promoter; and

(d) an intron which is 5' of said DNA encoding said desired nucleic acid product,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly.

13. A recombinant vector comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of

said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly.

14. A recombinant vector comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter;

(c) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety; and

(d) an intron,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly.

15. A host cell comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly.

16. A host cell of Claim 15 further comprising an intron, wherein the intron is 5' of the DNA of (b).

17. A host cell comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly.

18. A host cell of Claim 17 further comprising an intron, wherein the intron is 5' of the DNA of (b).

19. A viral vector including in its genome:

(a) a promoter;

(b) a nucleotide sequence encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) a nucleotide sequence encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the nucleotide sequence of (b) and the nucleotide sequence of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly.

20. A viral vector of Claim 19 additionally including in its genome an intron, wherein the intron is 5' of the nucleotide sequence of (b).

21. A viral vector including in its genome:

(a) a promoter;

(b) a nucleotide sequence encoding a desired nucleic acid product operably linked to said promoter; and

(c) a nucleotide sequence encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the nucleotide sequence of (b) and the nucleotide sequence of (c) produces a RNA molecule comprising said aptamer-self-

cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable cleaving said RNA molecule intramolecularly.

22. A viral vector of Claim 21 additionally including in its genome an intron, wherein the intron is 5' of the nucleotide sequence of (b).

23. A method of inducing expression of a desired nucleic acid product in a host cell comprising introducing into the host cell an agent which inhibits cleavage of a self-cleaving RNA motif, wherein the host cell comprises:

(a) a promoter;

(b) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly.

24. The method of Claim 23 wherein the agent is an antibiotic.

25. A method of modulating expression of a desired nucleic acid product in a host cell comprising introducing into the host cell an effector which binds an aptamer moiety of an aptamer-self-cleaving RNA motif complex, wherein the host cell comprises:

(a) a promoter;

(b) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly.

26. The method of Claim 25 wherein the host cell further comprises an intron which is 5' of the DNA of (b).

27. A method for producing a transgenic nonhuman animal whose cells contain a DNA construct comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding a self-cleaving RNA motif which is downstream of said promoter, wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly,

comprising introducing the DNA construct into a germ cell of a nonhuman animal or a germ cell of an ancestor of the animal.

28. The method of Claim 27 wherein the DNA construct further comprises an intron, wherein the intron is 5' of the DNA of (b).

29. A method for producing a transgenic nonhuman animal whose cells contain a DNA construct comprising:

(a) a promoter;

(b) DNA encoding a desired nucleic acid product which is operably linked to said promoter; and

(c) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety, wherein transcription of the DNA of (b) and the DNA of (c) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly,

comprising introducing the DNA construct into a germ cell of a nonhuman animal or a germ cell of an ancestor of the animal.

30. The method of Claim 29 wherein the DNA construct further comprises an intron, wherein the intron is 5' of the DNA of (b).

31. A method of inducing expression of a desired nucleic acid product in a cell, comprising the steps of:

(a) introducing into the cell a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly; and

(b) introducing into the cell an agent which is capable of inhibiting cleavage of the self-cleaving RNA.

32. The method of Claim 31 wherein the agent is an antibiotic.

33. The method of Claim 31 wherein the DNA construct further comprises an intron, wherein the intron is 5' of the DNA of (2).

34. A method of inducing expression of a desired nucleic acid product in a cell, comprising the steps of:

(a) introducing into the cell a viral vector including in its genome:

(1) a promoter;

(2) a nucleotide sequence encoding the desired nucleic acid product operably linked to said promoter; and

(3) a nucleotide sequence encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the nucleotide sequence of (2) and the nucleotide sequence of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid

product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly; and

(b) introducing into the cell an agent which is capable of inhibiting cleavage of the self-cleaving RNA.

35. A method of modulating expression of a desired nucleic acid product in a cell, comprising the steps of:

(a) introducing into the cell a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly; and

(b) introducing into the cell an effector which is capable of binding to the aptamer moiety of the complex.

36. The method of Claim 35 wherein the DNA construct further comprises an intron, wherein the intron is 5' of the DNA of (2).

37. A method of modulating expression of a desired nucleic acid product in a cell, comprising the steps of:

(a) introducing into the cell a viral vector including in its genome:

(1) a promoter;

(2) a nucleotide sequence encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) a nucleotide sequence encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the nucleotide sequence of (2) and the nucleotide sequence of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly; and

(b) introducing into the cell an effector which is capable of binding to the aptamer moiety of the complex.

38. A method of inducing expression of a desired nucleic acid product in an individual, comprising the steps of:

(a) obtaining cells from the individual under conditions appropriate for cell growth and cell division;

(b) introducing into the cells obtained in step (a) a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly;

(c) returning the cells produced in step (b) to the individual; and

(d) administering to the individual an agent which is capable of inhibiting cleavage of the self-cleaving RNA motif.

39. The method of Claim 38 wherein the agent is an antibiotic.

40. The method of Claim 38 wherein the DNA construct further comprises an intron, wherein the intron is 5' of the DNA of (2).

41. A method for modulating expression of a desired nucleic acid product in an individual, comprising the steps of:

(a) obtaining cells from the individual under conditions appropriate for cell growth and cell division;

(b) introducing into the cells a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such

that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety, wherein transcriptin of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly;

(c) returning the cells produced in step (b) to the individual; and

(d) administering to the individual an effector which is capable of binding to the aptamer moiety of the complex.

42. A method of expressing a desired nucleic acid product in an individual comprising the steps of:

(a) introducing into the individual cells which comprise a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an agent which is capable of inhibiting cleavage of the self-cleaving RNA motif.

43. A method of expressing a desired nucleic acid product in an individual comprising the steps of:

(a) introducing into the individual cells which comprise a viral vector including in its genome:

(1) a promoter;

(2) a nucleotide sequence encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) a nucleotide sequence encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the nucleotide sequence of (2) and the nucleotide sequence of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an agent which is capable of inhibiting cleavage of the self-cleaving RNA motif.

44. A method of modulating expression of a desired nucleic acid product in an individual comprising the steps of:

(a) introducing into the individual cells which comprise a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such

that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety, wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an effector which is capable of binding to the aptamer moiety of the aptamer-self-cleaving RNA motif complex.

45. A method of modulating expression of a desired nucleic acid product in an individual comprising the steps of:

(a) introducing into the individual cells which comprise a viral vector including in its genome:

(1) a promoter;

(2) a nucleotide sequence encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) a nucleotide sequence encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the nucleotide sequence of (2) and the nucleotide sequence of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif

moiety of said complex is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an effector which is capable of binding to the aptamer moiety of the aptamer-self-cleaving RNA motif complex.

46. A method of identifying an effector which is capable of binding to a desired aptamer moiety comprising the steps of:

(a) introducing into host cells a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding a reporter which is operably linked to said promoter; and

(3) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising the desired aptamer moiety and a self-cleaving RNA motif moiety, wherein said desired aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be inhibited by binding of an effector to the aptamer moiety,

wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said reporter, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly;

(b) introducing into the host cells an agent to be assessed for its ability to bind to the desired aptamer moiety under conditions appropriate for expression of the reporter; and

(c) assaying reporter activity,

wherein detection of reporter activity identifies the agent as an effector which binds to the desired aptamer moiety.

47. A method of screening for an agent which is capable of inhibiting the catalytic activity of a self-cleaving RNA motif comprising a random stem loop II, comprising the steps of:

(a) introducing into host cells a DNA construct which comprises:

(1) a promoter;

(2) DNA encoding a reporter which is operably linked to said promoter; and

(3) DNA encoding a self-cleaving RNA motif modified to comprise a random sequence at a position in said self-cleaving RNA motif capable of modulating the cleaving activity of said self-cleaving RNA,

wherein the DNA of (2) and the DNA of (3) are downstream of the promoter;

(b) introducing into the host cells an agent to be assessed for its ability to inhibit the catalytic activity of a self-cleaving RNA motif comprising the random sequence under conditions appropriate for expression of the reporter; and

(c) assaying reporter activity,

wherein detection of reporter activity identified the agent as an agent which inhibits the catalytic activity of the self-cleaving RNA motif comprising a random sequence.

48. A method of expressing a desired nucleic acid product in an individual comprising the steps of:

(a) introducing into the individual a viral vector including in its genome:

(1) a promoter;

(2) a nucleotide sequence encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) a nucleotide sequence encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the nucleotide sequence of (2) and the nucleotide sequence of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an agent which is capable of inhibiting cleavage of said self-cleaving RNA motif.

49. A method of expressing a desired nucleic acid product in an individual comprising the steps of:

(a) introducing into the individual a DNA construct comprising:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding a self-cleaving RNA motif which is downstream of said promoter,

wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said self-cleaving RNA motif and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an agent which is capable of inhibiting cleavage of the self-cleaving RNA motif.

50. A method of modulating expression of a desired product in an individual comprising the steps of:

(a) introducing into the individual a viral vector including in its genome:

(1) a promoter;

(2) a nucleotide sequence encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) a nucleotide sequence encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety,

wherein transcription of the nucleotide sequence of (2) and the nucleotide sequence of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an effector which is capable of binding to the aptamer moiety of the aptamer-self-cleaving RNA motif complex.

51. A method of modulating expression of a desired product in an individual comprising the steps of:

(a) introducing into the individual a DNA construct comprising:

(1) a promoter;

(2) DNA encoding the desired nucleic acid product which is operably linked to said promoter; and

(3) DNA encoding an aptamer-self-cleaving RNA motif complex which is downstream of said promoter, said complex comprising an aptamer moiety and a self-cleaving RNA motif moiety, wherein said aptamer moiety is in said complex in a position such

that the cleaving activity of said self-cleaving RNA motif moiety can be modulated by binding of an effector to the aptamer moiety, wherein transcription of the DNA of (2) and the DNA of (3) produces a RNA molecule comprising said aptamer-self-cleaving RNA motif complex and mRNA encoding said desired nucleic acid product, wherein said self-cleaving RNA motif moiety of said complex is capable of cleaving said RNA molecule intramolecularly; and

(b) administering to the individual an effector which is capable of binding to the aptamer moiety of the aptamer-self-cleaving RNA motif complex.

Strategy for regulation of gene expression *via* incorporation of self cleaving RNA motifs into a mammalian mRNA

Figure 1A

Structure of ribozymes used in study

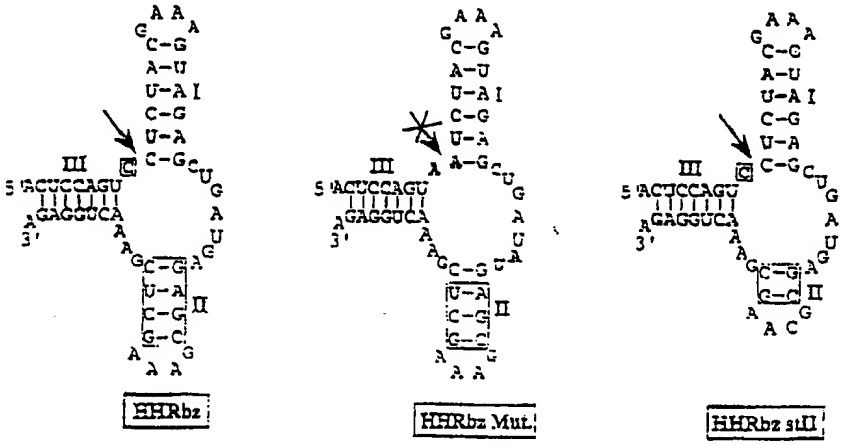


Figure 1B

Cleavage of mRNA prevents gene expression

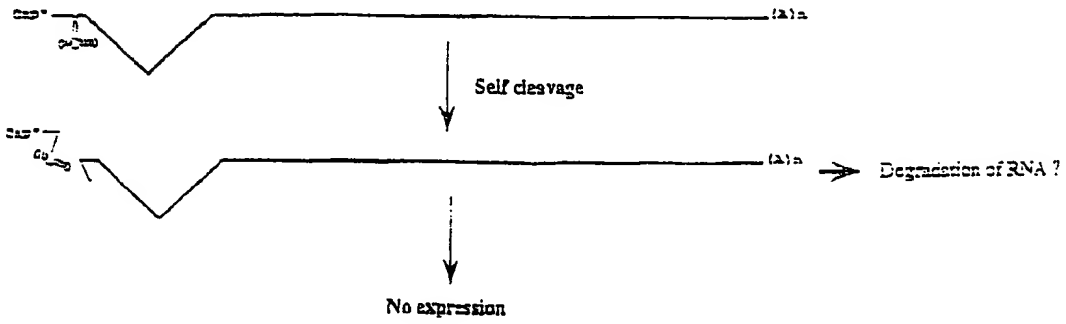
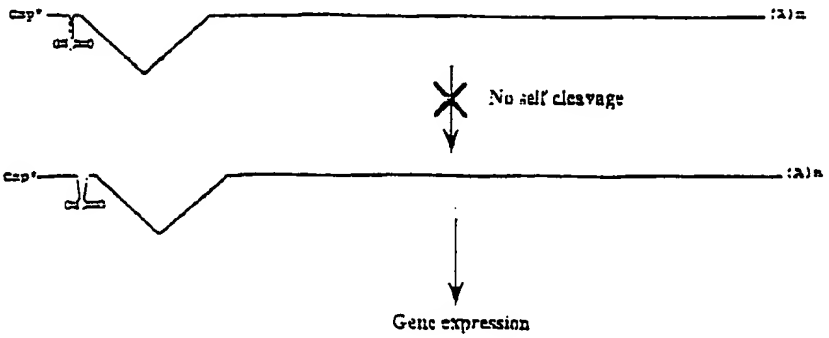


Figure 1C

Inhibition of self-cleavage by small molecule activates gene expression



Evaluation of the activity of ribozymes inserted into different locations within a transcriptional unit

Figure 2A

Sites of insertion of ribozymes in pMD vector

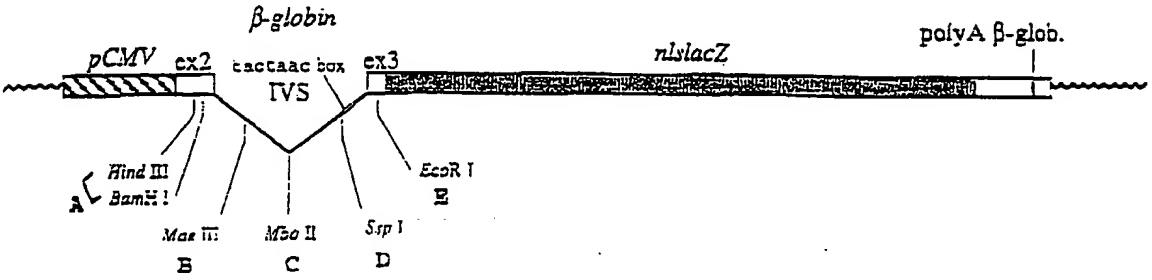
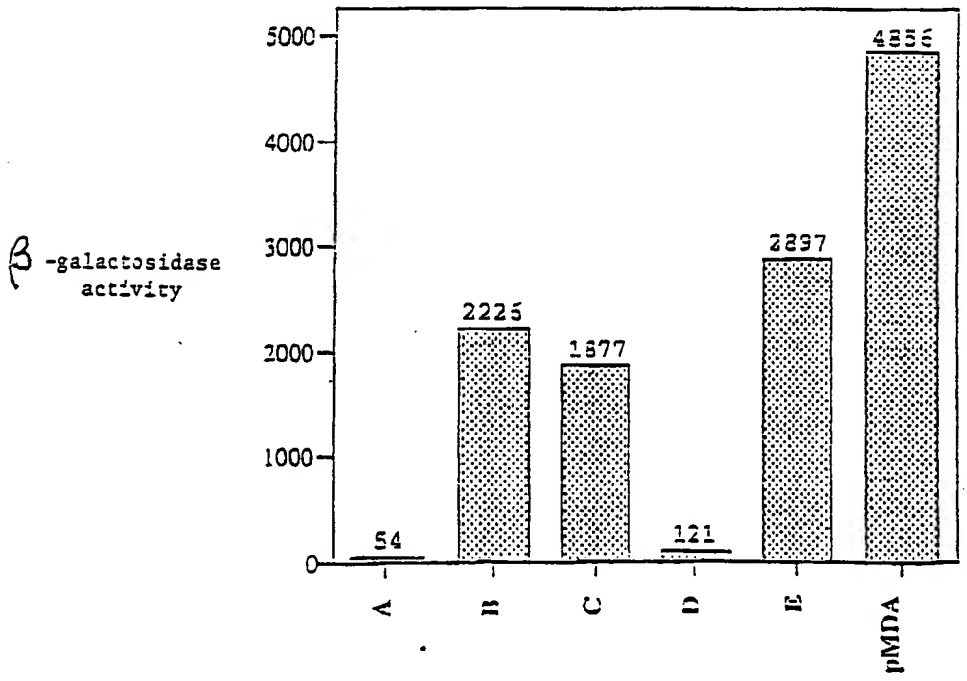


Figure 2B

Effect of specific placement of ribozyme on gene expression in transiently transfected 293 cells



Induction of gene expression by Chlortetracycline or Neomycin after transient transfection of 293T cells

Figure 3A

Chlortetracyline

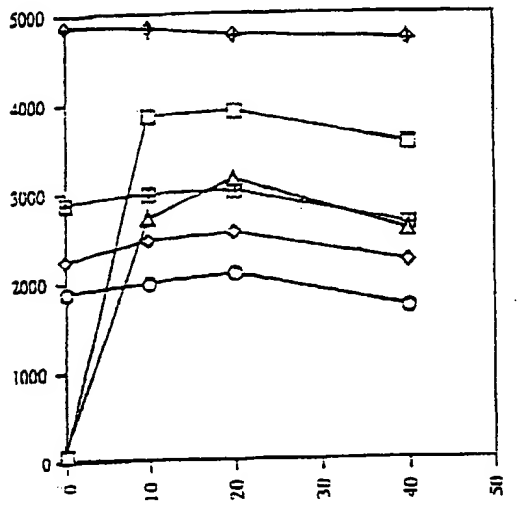
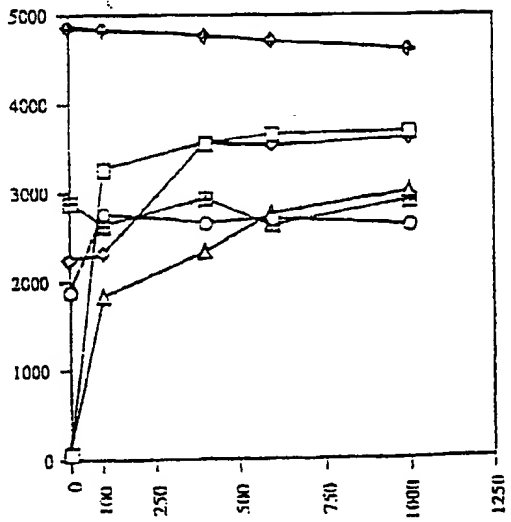


Figure 3B

Neomycin



Concentration of antibiotic (µg/ml)

HinD III- BamH I

Mac III

Mbo II

Ssp I

EcuR I

pMDA

Activation of gene expression of ribozyme containing constructs by addition of common antibiotics

Figure 4A

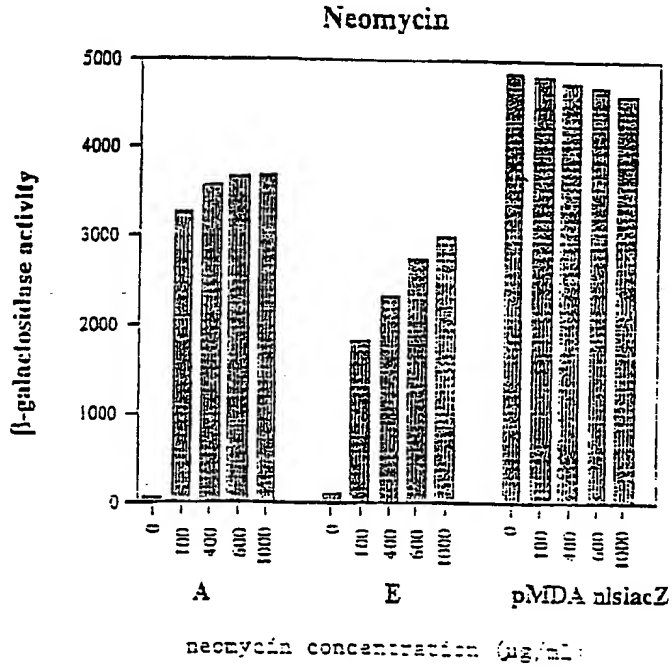


Figure 4B

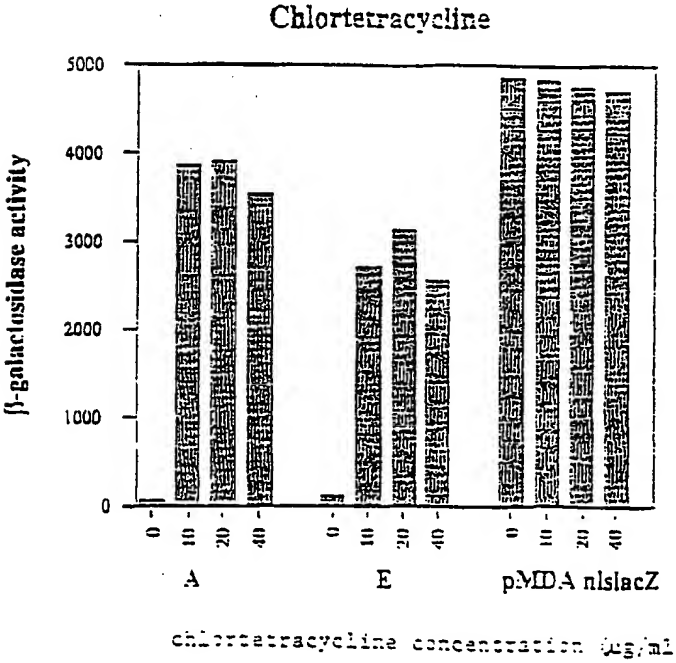


Figure 5A

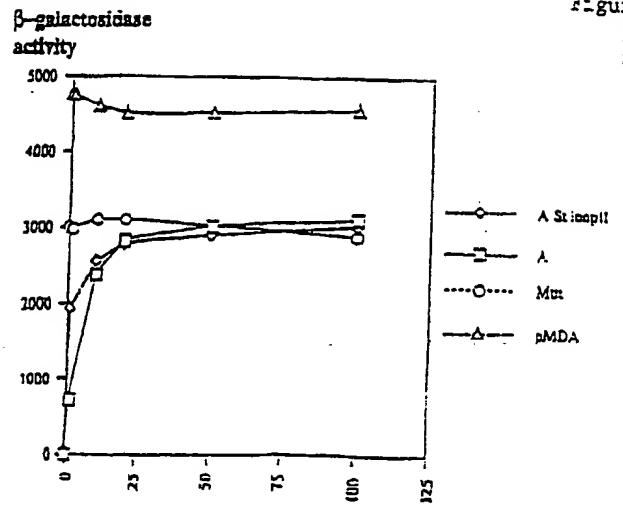
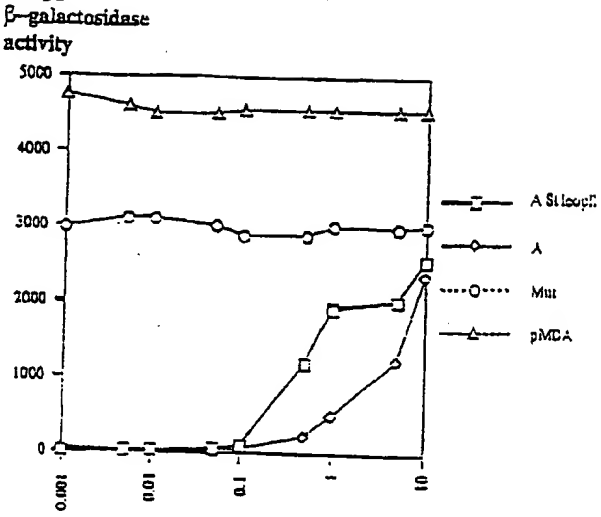


Figure 5B



Chlortetracycline concentrations (μg/ml)

Figure 6

Rbz insertion at the polyA site.

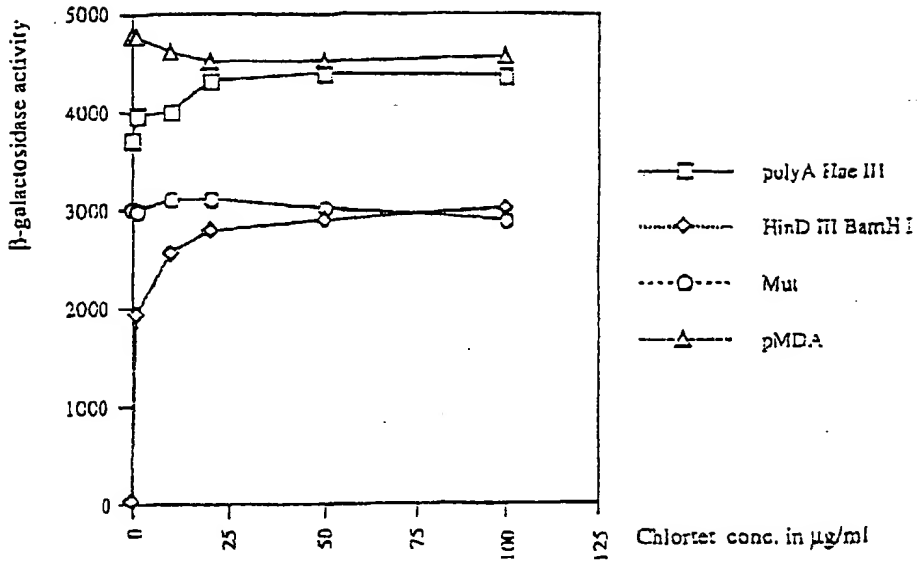
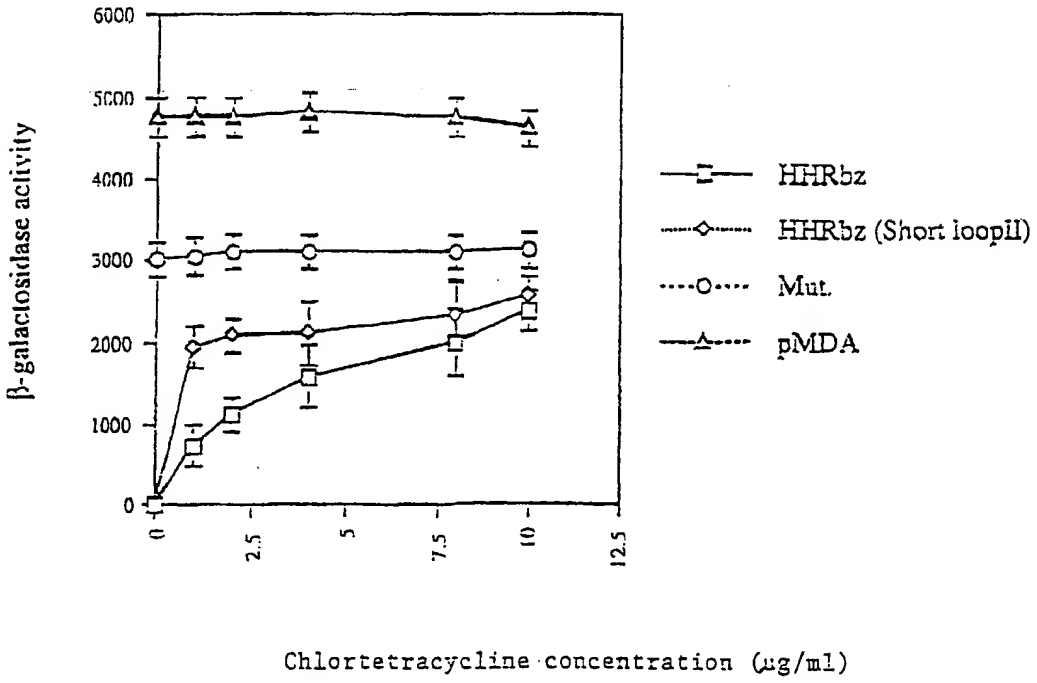


Figure 7

Effect of loop II length on induction of gene expression by Chlortetracycline

Exp. I



Cells stably expressing ribozyme-containing constructs show no detectable activity in absence of antibiotic

Figure 8A

Figure 8B

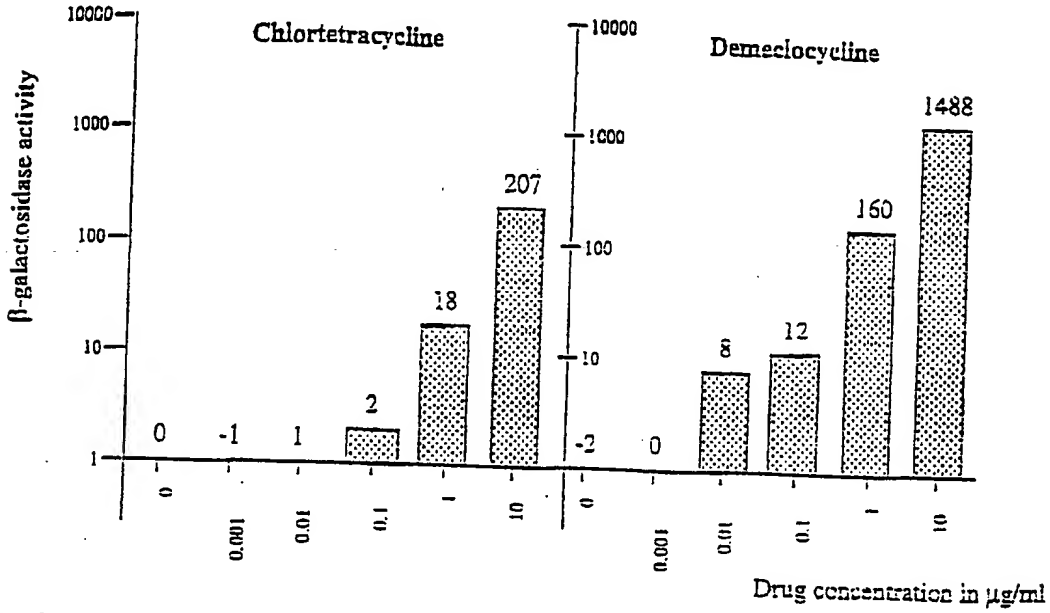


Figure 8C

Figure 8D

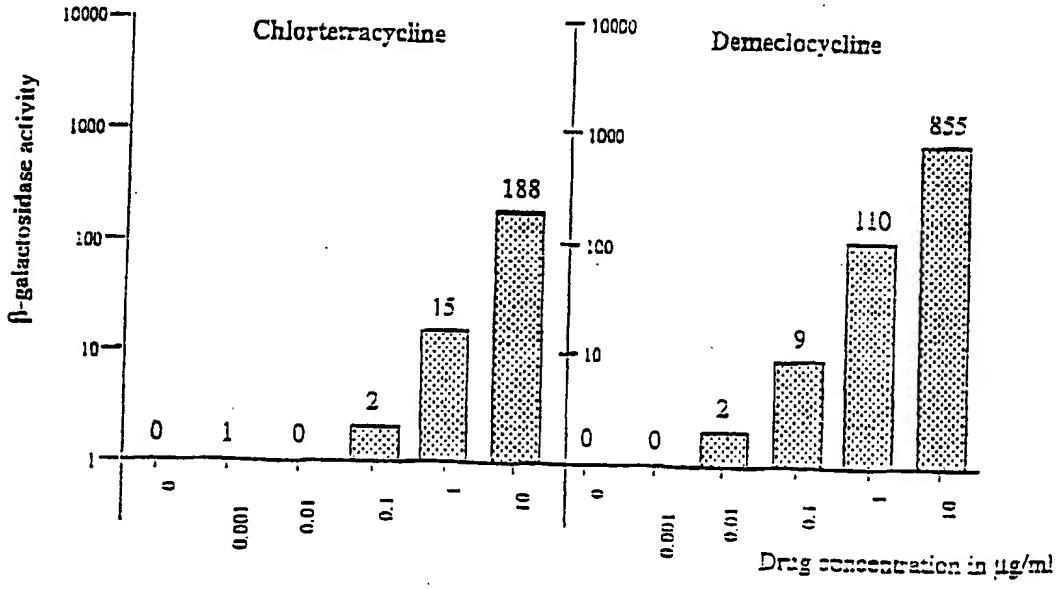


Figure 9A

Cleavage of mRNA prevents gene expression

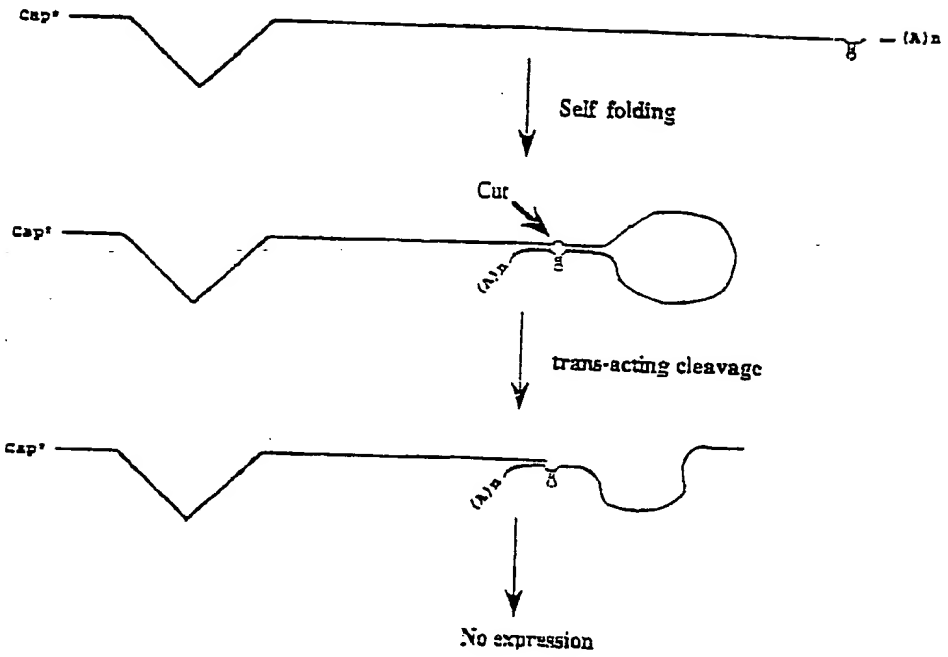
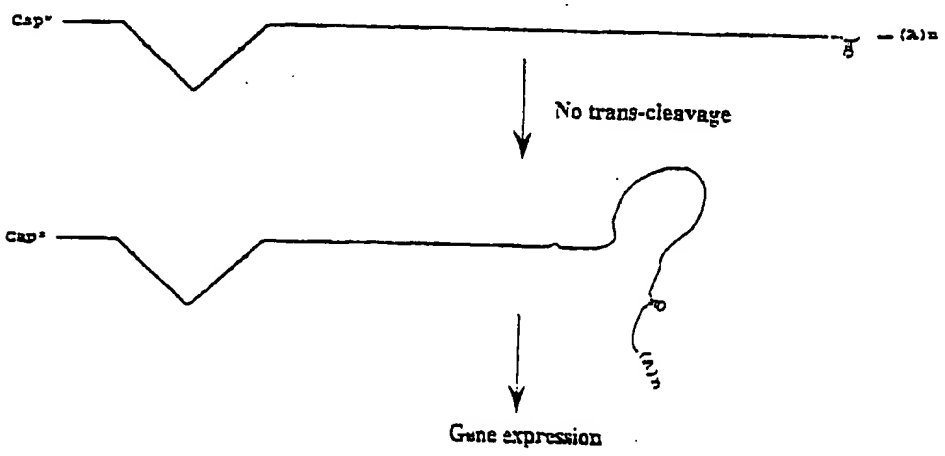


Figure 9B

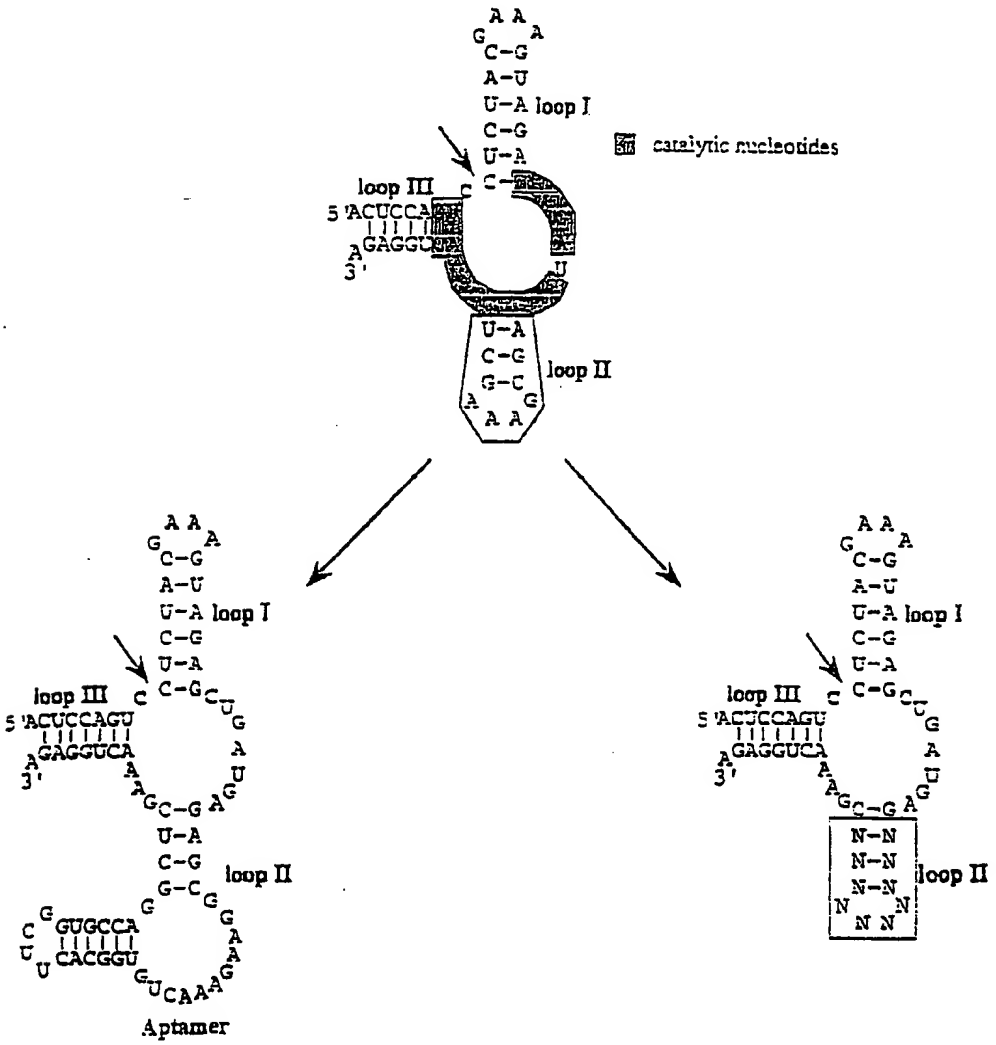
Inhibition of trans-acting cleavage by small molecule activates gene expression



Generation of unique small regulators of gene expression

Figure 10

Hammerhead ribozyme catalytic function is sensitive to changes in loop II stem and sequences



Approach I:
Grafting of RNA aptamer sequences onto hammerhead ribozymes by either rational design or in vitro evolution

Approach II:
Generation of random loop II sequences followed by high throughput screen of chemical libraries

INTERNATIONAL SEARCH REPORT

Int. Sional Application No
PCT/US 99/24781

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/63 C12N15/67 C12N15/86 C12N15/10 C12Q1/68
A01K67/027 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 43993 A (UNIV YALE) 8 October 1998 (1998-10-08) page 7, line 16 - line 30 page 11, line 18 -page 12, line 9 page 16, line 15 -page 18, line 30	1-23,25, 26,31, 33, 35-37, 41-45, 47-51
Y	examples claims 31-38	11-22, 24, 27-30, 32,39

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2000	10/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WERSTUCK AND GREEN: "CONTROLLING GENE EXPRESSION IN LIVING CELLS THROUGH SMALL MOLECULE-RNA INTERACTIONS" SCIENCE, vol. 282, 9 October 1998 (1998-10-09), pages 296-298, XP002113355 ISSN: 0036-8075 the whole document	24,32,39
Y	ORY D S ET AL: "A STABLE HUMAN-DERIVED PACKAGING CELL LINE FOR PRODUCTION OF HIGH TITER RETROVIRUS/VESICULAR STOMATITIS VIRUS G PSEUDOTYPES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 21, 15 October 1996 (1996-10-15), pages 11400-11406, XP002030515 ISSN: 0027-8424 cited in the application the whole document	11-22
Y	WO 95 28412 A (GUSTAFSSON KENTH T ;INST OF CHILD HEALTH (GB); BAETSCHER MANFRED W) 26 October 1995 (1995-10-26) claims	27-30
X	WO 94 13791 A (INNOVIR LAB INC) 23 June 1994 (1994-06-23) figure 4; example 3 claims	1,6,11, 13,15, 17,23, 25,31, 35,38, 41,42, 44, 47-49,51
X	WO 98 08974 A (TIKOCHINSKI YARON ;ASHER NATHAN (IL); INTELLIGENE LTD (IL); ELLING) 5 March 1998 (1998-03-05) page 7 -page 19 claims; examples	1,6,11, 13,15, 17,25, 35,41, 44,46-51
A	ARAKI MICHIRO ET AL: "Allosteric regulation of a ribozyme activity through ligand-induced conformational change." NUCLEIC ACIDS RESEARCH , vol. 26, no. 14, 15 July 1998 (1998-07-15), pages 3379-3384, XP002134018 ISSN: 0305-1048	

-/—

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MURRAY, J. & ARNOLD, J.: "Antibiotic interactions with the hammerhead ribozyme: tetracyclines as a new class of hammerhead inhibitor" BIOCHEMICAL JOURNAL, vol. 317, 1996, pages 855-860, XP002134019 ISSN: 0264-6021 cited in the application	
A	DENISON C. ET AL.: "Small-molecule-based strategies for controlling gene expression." CHEM BIOL 1998 JUN;5(6):R129-45, XP000892765	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 24781

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 23-26,31-37 (as far as in vivo methods are concerned) and claims 38-45, 48-51 are directed to a method of the human/animal body, the search has been carried out and based of the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 99/24781

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9843993 A	08-10-1998	AU 6591798 A	22-10-1998
WO 9528412 A	26-10-1995	AU 1850599 A	29-04-1999
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		JP 10504442 T	06-05-1998
WO 9413791 A	23-06-1994	AU 679525 B	03-07-1997
		AU 6653994 A	04-07-1994
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WO 9808974 A	05-03-1998	AU 3862097 A	19-03-1998
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		EP 0970241 A	12-01-2000
		WO 9841654 A	24-09-1998

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